

HIGH PHYLOGENETIC DIVERSITY OF BACTERIA IN THE AREA OF PREHISTORIC PAINTINGS IN MAGURA CAVE, BULGARIA

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Abstract: Magura Cave, situated in the northwest of Bulgaria and containing prehistoric paintings, is a famous tourist site. The present study is the first report on bacterial diversity in guano paintings in a Bulgarian cave using molecular methods. We identified 68 taxa, which is an unusually high number for oligotrophic niches. They are affiliated with eight phyla representing more than half of the bacterial divisions identified in caves. As in many other caves, *Proteobacteria* dominated in this type of ecosystem (about 40%), followed by *Nitrospirae* (22.5%) and *Acidobacteria* (21.5%). Weakly represented were *Actinobacteria* (6.4%), *Chloroflexi* (3.2%), *Planctomycetes* (2.2%), *Firmicutes* (2.2%), and *Gemmatimonadetes* (2.2%). About one third of all DNA sequences recovered in this study were new. Some of them had more than 10% divergence from the closest neighbor, which suggests the existence of new taxa of at least genus level. Bacteria identified in the community expressed various types of metabolism; lithoautotrophic, organotrophic, and methylotrophic.

INTRODUCTION

Karst landscapes and caves are formed in soluble rock over millions of years. In the last two centuries, traces of prehistoric life were found in many caves across the continents. The interiors of caves have stable environmental conditions such as temperature, and this has favored their occupation by prehistoric people. Several caves have authenticated paintings connected with religious and everyday habits, some of them representing real masterpieces of ancient art (Schabereiter-Gurtner et al., 2002c; Pike et al., 2012). UNESCO has recognized such caves as a valuable part of the world's cultural heritage. Caves with prehistoric images are found in France, Spain, and Italy. Until recently, the paintings found in Chauvet Cave, France were accepted as the oldest in Europe, but recent studies using radioactive decay rates of uranium revealed that the paintings in El Castillo Cave in Spain date back to at least 40,800 years, making them the oldest known cave art in Europe, five to ten thousand years older than previous examples from France (Pike et al., 2012). The cave microbiota have been well characterized in several Spanish caves, including Tito Bustillo (Schabereiter-Gurtner et al., 2002b), Altamira (Schabereiter-Gurtner et al., 2002a), and La Garma and Llonín (Schabereiter-Gurtner et al., 2004). Rock paintings are found in several Bulgarian caves, with Magura Cave being the largest and having the best-conserved images. These ancient paintings are multi-layered, dating from the Neolithic and Eneolithic Ages to the beginning of the early Bronze Age (Stoytchev, 1994).

Prehistoric paintings in caves have been made predominantly with inorganic pigments including hematite (red), limonite (yellow), manganese oxide, and charcoal (black)

(Onac and Forti, 2011). There are many examples of prehistoric paintings with inorganic pigments, including black charcoal images in Chauvet Cave (Sadier et al., 2012) and ochre images in Spanish caves Tito Bustillo, Monte Castillo (Iriarte et al., 2009), Altamira (Schabereiter-Gurtner et al., 2002a), and El Castillo, and the French cave Niaux (Valladas et al., 1992). Two pigments were used in the Cave of Bats, Zuheros, Spain, red ones composed mainly of hematite (iron oxide) mixed with calcite and black ones of undetermined origin (Urzi et al., 2010). To the best of our knowledge, the paintings in only three caves have been created using fossilized bat guano: Magura Cave and Baylovo Cave, Bulgaria (Stoytchev, 2005), and a part of the paintings in Grotta dei Cervi, Italy (Laiz et al., 2000).

Despite its archaeological importance, the knowledge of the microbiology of caves is still incomplete. Although geomicrobiological environmental investigations have been widely published, a significant increase in biospeleological research has occurred within the last two decades (Urzi et al., 2002).

Most caves offer similar conditions for microbial growth, including a relatively constant temperature of 8 to 12 °C, absence of light (preventing photosynthesis), and sparse nutrients. The relatively constant conditions ensure the stability of microbial communities, which is why karst caves can be considered as a long-term home for the same microorganisms. Without photosynthesis, caves are deprived

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of the main energy supporting life on the earth's surface. To overcome those close-to-starvation conditions in caves, selfish competition for food is replaced by cooperative and mutualistic association (Barton and Jurado, 2007). According to Canaveras et al. (2001), caves are usually oligotrophic environments where primary production depends on autotrophic bacteria, and well-established autotrophic communities can support the growth of several chemoorganotrophic microbes (Urzi et al., 2010). Although many authors accept that autochthonic organic carbon is often produced from chemolithotrophic primary productivity in such ecosystems (Sarbu et al., 1996; Schabereiter-Gurtner et al., 2003, 2004; Opsahl and Chanton, 2006; Chen et al., 2009), others point out that wind, flowing and dripping water, or guano can provide organic material (Culver and Pipan, 2009).

According to Lehman (2007), microbial communities associated with different karst zones are distinct from each other, as supported by, for example, a domination of *Firmicutes* in Zuheros Cave, Spain (Urzi et al., 2010) or *Streptomyces* in Grotta dei Cervi, Italy (Laiz et al., 2000). On the other hand, some types of microbes are reported to be widespread in caves. Comparing five caves, Pronk et al. (2009) concluded that *Delta Proteobacteria*, *Acidobacteria*, and *Nitrospira* are specific cave groups. Indeed, the presence of these bacteria in caves has been mentioned by several authors (Schabereiter-Gurtner et al., 2002a, 2004; Pašić et al., 2010). Shabarova and Pernthaler (2009) suggested that three *Beta Proteobacteria* groups (families *Oxalobacteraceae*, *Methylophilaceae*, and *Comamonadaceae*) point toward a highly specialized endemic subsurface microflora. Consideration needs to be given to the possibility of contamination from drip waters or human presence for some pathogenic or enteric bacteria like *Escherichia coli*, *Enterococcus*, and *Staphylococcus* (Bastian et al., 2009; Kelly et al., 2009).

Among the less well known ecological zones on earth, caves located in Eastern European countries still present an opportunity for searching for taxa that could represent novel biotechnological resources. Although Magura Cave is very popular as a tourist site, its bacterial diversity had not been investigated. This report is the first culture-independent investigation on the bacterial structure of the microbial community from the area of the prehistoric paintings in Magura Cave.

MATERIALS AND METHODS

STUDY SITE AND SAMPLE COLLECTION

Bacterial community samples were collected from the prehistoric paintings in the Gallery, Magura Cave, northwestern Bulgaria (E 22°34'54.84", N 43°43'41.16"), located close to the village of Rabisha. The total length of Magura Cave exceeds 2.5 km, comprising an area of 28,600 m². The cave offers a rich collection of geological formations of all shapes and sizes—stalactites, stalagmites,



Figure 1. Some of the paintings in the Gallery, Magura Cave, including solar calendar and dancing figures.

columns, cave pearls, and flows of moonmilk. Remnants of prehistoric life, traces of settlements, and rock drawings have been found in the cave. One of the caverns, the Gallery, contains prehistoric paintings carved into the walls and decorated with bat guano. The Gallery is located on a branch off of one of the main rooms, the Landslip, and the isolated location has a constant temperature of 12 °C and a humidity of about 94%. It is the highest part of the cave, is 240 m long and up to 24 m high, and has an area of 3750 m². The drawings represent religious ceremonies, deities, and hunting scenes and include dancing male and female figures, hunting men, people wearing masks, a large variety of animals, and also suns, stars, tools, and non-figurative, symbolic, and abstract elements. A solar calendar from the late Neolithic found there is the earliest solar calendar discovered in Europe (Fig. 1). Magura Cave was recognized as a natural landmark in May 1960 and placed on the Tentative List for consideration as a World Heritage Site by UNESCO in 1984. The cave was opened for visitors in 1961, but the Gallery was open only for five years (2002–2007).

Twelve samples were collected from the areas of different painting groups along the whole length of the Gallery wall (105 m) at locations separated by 5 to 11 m. About one gram was scraped from the area of a painting or the rock within 2 cm of it with sterile scalpels under the supervision of the responsible archeologist from the local museum. The samples were placed in sterile tubes and transported to the laboratory in an insulated bag. All samples were mixed together and vortexed immediately after delivery to the laboratory and considered as a common sample. The procedure of total DNA isolation was initiated immediately using 10 g of the common sample.

The mineral composition of the rock in the area of the paintings was determined to be mainly limestone (CaCO₃,

74.02%), with saltpeter (KNO₃, 7.05%), gypsum (CaSO₄·2H₂O, 6.25%), hematite (Fe₂O₃, 5.05%), apatite (Ca₅(PO₄)₃(F,Cl,OH), 3.08%), calcium monohydrogen phosphate (CaHPO₄·2H₂O, 1.45%) taranakite (K₃Al₅[(PO₃OH)₃]PO₄]·18H₂O, 1.28%), and todorokite (Mn²⁺, Ca,Mg)Mn₃⁴⁺O₇·H₂O, 1.25%) (Benderev, 2006).

DNA EXTRACTION, PCR AMPLIFICATION AND CONSTRUCTION OF BACTERIAL 16S rRNA GENE LIBRARY

Total DNA was extracted from the common sample as described by Selenska-Pobell et al. (2001) with some modifications. A sample (10.0 g fresh weight) was measured aseptically and used for further analysis. The sample material was suspended in 10 ml of 0.12 M sodium phosphate buffer. Lysis of the cells was achieved after adding sodium dodecyl sulfate (final concentration 2%), NaCl (0.5 M), and PEG 6000 (20%). The protocol for extraction of a total-community DNA encompassed three cycles of freezing and thawing (respectively -80 °C and 96 °C), chemical lysis in an extraction buffer, and a proteinase K step. The crude DNA was purified with AXG-100 Nucleobond cartridges (Machery-Nagel, Duren, Germany) following the manufacturer's instructions. The eluate was precipitated by 0.7 volumes of ice-cold isopropanol. The integrity of the DNA was checked by horizontal electrophoresis in 1% agarose (Sigma) gel and visualized with ethidium bromide (0.5 µg/l).

Community ribosomal DNAs were amplified from 1 to 50 ng of bulk DNA in reactions containing (as final concentrations) 1× PCR buffer, 2 mM CaCl₂, 4× 200 µM deoxynucleoside triphosphates, 400 nM each forward and reverse primer, 0.5 U Taq polymerase (GenetBio, Korea). Two universal primers for domain *Bacteria* were used: 8F (EUB008, Hicks et al., 1992), and 1492R (EUB1492, Kane et al., 1993). Reaction mixtures were incubated in a BioRad thermal cycler (model T100) for an initial denaturation at 94 °C for 3 min followed by 30 cycles of 94 °C for 0.5 min, 55 °C for 0.5 min, and 72 °C for 1 min, and final extension at 72 °C for 5 min.

For characterization of the bacterial population, a gene library was constructed of the amplified products. The PCR products were cloned in *E. coli* JM 109 using the pJet1.2 cloning kit (Fermentas) according to manufacturer's instructions. Cloned fragments were reamplified using the primers pJet1.2 forward and pJet1.2 reverse located in the vector and surrounding the inserted PCR fragment.

RESTRICTION FRAGMENT LENGTH POLYMORPHISM (RFLP) ANALYSIS

PCR-amplified bacterial inserts were digested using Msp I followed by digestion with Hae III (1 U each) according to the manufacturer's instructions (Fermentas). Restriction profiles were analyzed using 2% agarose gel electrophoresis. The different banding patterns were noted, and clones were

grouped according to their RFLP patterns. The sequence of one clone from each group was determined.

16S rRNA GENE SEQUENCING AND ANALYSIS

16S rRNA gene sequences were determined with an Applied Biosystems model 373A DNA sequencer by using the ABI PRISM cycle sequencing kit (Macrogen, Korea), where they were reamplified by using 8F primer. Those of the clones that showed less than 97% similarity to the closest relative after sequencing with the forward primer were additionally sequenced with the reverse primer 1492R to establish if the full gene sequences were new ($\geq 3\%$ evolutionary distance of the full gene).

The degree to which RFLP types covered the diversity in the sample was measured with the Shannon index (e.g., Hill et al. (2003): $H = -\sum_i (p_i \ln(p_i))$), where p_i is the relative frequency of the i th in the examined clones and \ln is the natural logarithm.

The sequences obtained were checked for potential chimeric artifacts using the CHIMERA_CHECK program from RDPII on the Ribosomal Database web site (<http://rdp8.cme.msu.edu/html/analyses.html>). The sequences were compared to the closest relatives in the NCBI GenBank database using BLASTn (Altschul et al., 1990) and Ribosomal Database Project resources (Maidak et al., 1994) to determine their close relatives and approximate phylogenetic affiliations. Phylogenetic analyses were conducted using MEGA version 4.0 (Tamura et al., 2007) and the Neighbor-joining method (Saitou and Nei, 1987). The 16S rRNA gene sequences of the sequenced clones have the Gen Bank accession numbers HE653817 to HE653888.

RESULTS

CLONE LIBRARY

To determine the structure of the microbial community in the Gallery in Magura Cave, we analyzed the sequences of 16S rRNA genes obtained by PCR amplification followed by cloning. The isolated 126 correct clones containing 16S rRNA gene were screened by RFLP to identify unique types for sequence determination. rDNA digestion resulted in three to ten bands with the discernible fragment size range of 50 to 1000 bp from each clone (data not shown). A total of 93 different rDNA groups were formed; 77 of them contained only one clone. More than 96% of the clone fragments sequenced with an 8F primer were longer than 900 bp. Sequences for which the similarity to the closest relative was less than 97% for the full gene sequence were defined as new. They represented 31.9% of all clone sequences. Sequences differing only slightly ($\leq 2\%$) were considered as a single relatedness group. Clone analysis revealed the presence of eight bacterial phyla. These were *Proteobacteria*, *Acidobacteria*, *Nitrospirae*, *Actinobacteria*, *Planctomycetes*, *Firmicutes*, *Chloroflexi*, and *Gemmatimonadetes*.

PROTEOBACTERIA

Thirty-seven of the clones in the library assigned to thirty restriction groups were related to *Proteobacteria* (Fig. 2), subphyla *Alpha*, *Beta*, *Gamma*, and *Delta*.

The taxonomic groups affiliated with *Alpha Proteobacteria* were represented by one clone for each group (nine clones, nine groups). Unlike all other groups, most of the sequences related to *Alpha Proteobacteria* had culturable microorganisms as closest neighbors (orders *Rhizobiales* and *Sphingomonadales*). Identified clones represented metabolic types methylotrophs (*Methylobacterium* and *Hyphomicrobium*) and heterotrophs (*Sphingomonas*). Aerobic metabolism was characteristic for all identified genera.

With respect to *Beta Proteobacteria*, nine clones were spread in six groups and corresponded to two orders, *Burkholderiales* (genera *Methylibium* and *Duganella*) and *Rhodocyclales* (family *Rhodocyclaceae*). More than half of the clones (five out of nine) were affiliated with the genus *Methylibium*, including facultative anaerobes, methylotrophs, or organotrophs with optimal growth at pH 6.5 and temperature 30 °C (Nakatsu et al., 2006). Bacteria from the genus *Duganella* are strict aerobes and also organotrophs. The microorganisms from the family *Rhodocyclaceae* prefer oligotrophic niches and have diverse metabolisms.

Most of the *Proteobacteria* clones referred to the subphylum *Gamma Proteobacteria* (fifteen clones spread in eleven groups). Only two of these sequences grouped together with culturable microorganisms from the genera *Nitrococcus* and *Enterobacter*; all others grouped together with unculturable sequences. Microorganisms from the genus *Nitrococcus* oxidize nitrites to nitrates and play a key role in nitrate cycling. Two of the sequences (HE653823 and HE653851) related to family *Chromatiaceae* are novel. They have 92 to 95% similarity to the closest neighbor. *Chromatiaceae* are sulfur bacteria, oxidizing sulfides.

Four clones distributed in four different groups corresponded to *Delta Proteobacteria* representing anaerobic sulfate-reducing bacteria. All phylogenetic neighbors of the clones from this subphylum were unculturable bacteria. Three of the four sequences in this subphylum are new, with similarity 93 to 96% to the unculturable sulfate-reducing bacteria from the order *Syntrophobacterales* and saprophytic order *Myxococcales*. The role of the *Delta Proteobacteria* is hypothetical due to the low phylogenetic similarity. Sequences related to *Epsilon Proteobacteria* were not identified in Magura Cave.

NITROSPIRAE

All identified twenty-one sequences from this phylum were referred to the genus *Nitrospira*; nineteen of them had environmental sequences as closest neighbors (Fig. 3). Five clones were only distantly related, with similarity 88 to 95%, suggesting the existence of new taxa in the phylum. Sequence HE653833 showed 89% similarity with the closest unculturable neighbor and 88% with the culturable

Candidatus Nitrospira defluvii. One of the sequences (HE653821) had already been identified in another cave, Pajsarjeva jama, Slovenia (Pašić et al., 2010). Although a small number of culturable *Nitrospirae* are known, all of them are obligate chemolithotrophs.

ACIDOBACTERIA

Twenty of the clones spread in twelve groups were referred to the phylum *Acidobacteria* (Fig. 4). All identified sequences grouped together with environmental sequences, unlike the *Acidobacteria* taxa identified in La Garma Cave, Spain, which were closer to culturable representatives of the phylum (Schabereiter-Gurtner et al., 2004). *Acidobacteria* is a monophyletic phylum comprising eleven deeply branched groups (Zimmerman et al., 2005). Three of them (1, 3, and 7) were identified in Magura's Gallery. Half of the identified *Acidobacteria* sequences showed low similarity to the closest neighbors (92 to 95%), suggesting that the *Acidobacteria* community in Magura Cave differs from those described so far.

ACTINOBACTERIA

Actinobacteria was weakly presented by six clones spread in five groups (Fig. 4). All five sequences showed highest similarity with environmental sequences; two of them corresponded to order *Actinomycetales* and one to the family *Acidothermaceae*.

PHYLA PLANCTOMYCETES, FIRMICUTES, CHLOROFLEXI, AND GEMMATIMONADETES

Phyla weakly represented in the community (less than 10%) were *Chloroflexi* (3 clones, 3 groups), *Planctomycetes* (2 clones, 2 groups), *Firmicutes* (2 clones, 2 groups), and *Gemmatimonadetes* (2 clones, 1 group) (Table 1). One of the *Chloroflexi* sequences (HE653852) showed 92% similarity to the closest neighbor, and one of the *Planctomycetes* sequences (HE653854) showed 95% similarity.

DISCUSSION

The culture-independent approach to microbiological investigations of caves with prehistoric paintings has revealed an unusually complicated structure of bacterial communities (Gurtner et al., 2000; Schabereiter-Gurtner et al., 2002a, b; 2003; 2004) despite the commonly observed low degree of diversity for oligotrophic niches. Our phylogenetic analysis of the bacterial community in the painted Gallery in Magura Cave revealed the highest number of identified phylotypes (68) among all comparable caves. Despite the fact that the number of the analyzed clones (126) was higher than that for caves Llonín (27), La Garma (58), Altamira (21), and Tito Bustillo (41), a comparatively low diversity measure was estimated (Shannon index $H = 4.43$). This measure, which expressed the certainty of appearance of a random selected sequence, reflects the significant variety in the taxa in Magura Cave.

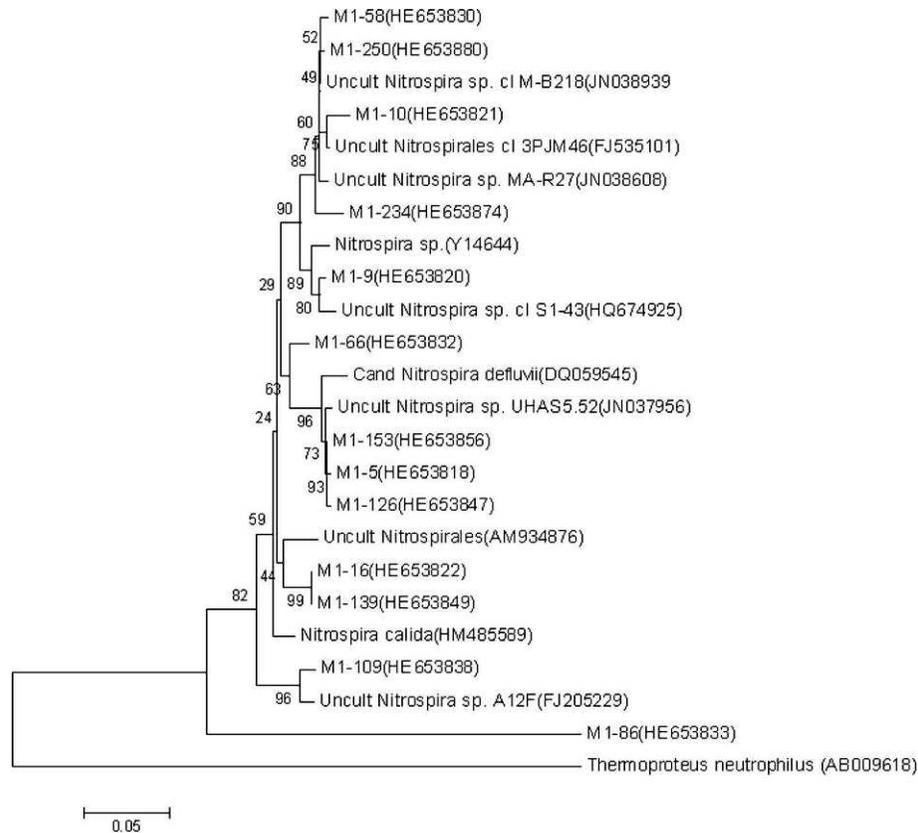


Figure 3. Neighbor-joining tree, calculated using evolutionary distances, based on *Nitrospirae* 16S rRNA sequence types from Magura Cave. Scale bar 5% substitutions in nucleotide sequence. Bootstrap values are shown at branching points (percentage of 1000 resamplings). Sequence accession numbers are given in parenthesis. *Thermoproteus neutrophilus* was used as the out-group.

Most of the sequences identified in Magura Cave are phylogenetically close to environmental sequences (57 out of the presented 68 phylotypes, 84%). A predominance of unculturable phylogenetic neighbors was also observed for Altamira, Spain (62%) (Schabereiter-Gurtner et al., 2002a), while unculturable and culturable neighbors were equally presented in the caves Llonín and La Garma, Spain (Schabereiter-Gurtner et al., 2004). Culturable neighbors of the sequences from the Magura Cave samples predominated only in the subphylum *Alpha Proteobacteria* and were fully absent in *Delta Proteobacteria*, *Acidobacteria*, *Actinobacteria*, *Planctomycetes*, *Chloroflexi*, *Firmicutes*, and *Gemmatimonadetes*. Branching of the sequences almost exclusively between unculturable bacteria confirms that a great variety of microorganisms remain undiscovered and uncharacterized in various natural microbial habitats (Ranjard et al., 2000). Our results confirm the wide spectrum of still unculturable bacteria and the importance of the culture-independent approach to understanding the whole bacterial diversity in caves.

Currently domain *Bacteria* comprises twenty-nine validly described taxonomic divisions (Ciccarelli et al., 2006), and according to Engel (2010) almost half of them

have been identified in caves. The retrieved sequences from Magura Cave corresponded to eight bacterial phyla (Fig. 5), more than a half of the divisions reported for caves. Investigations of the samples from five caves located in different places have revealed that the number of phyla represented varied from six in Altamira Cave (Schabereiter-Gurtner et al., 2002a) and Tito Bustillo (Schabereiter-Gurtner et al., 2002b) to seven in Llonín and eight in La Garma (Schabereiter-Gurtner et al., 2004) and Magura Cave. A general lack of phyla unique to a given cave was observed in the phyla presented in the compared caves. Distinct phylogenetic clusters are repeatedly detected in the caves, like *Proteobacteria*, *Nitrospirae*, and *Actinobacteria*. *Proteobacteria* was found to dominate in all compared caves (Table 2). Almost 40% of the sequences retrieved in Magura samples were identified with this phylum. Phyla *Nitrospirae* and *Acidobacteria* also were presented as dominant groups (more than 10%). Five other phyla (*Actinobacteria*, *Planctomycetes*, *Firmicutes*, *Chloroflexi* and *Gemmatimonadetes*) were weakly represented.

The highest similarity in the presented bacterial groups and their degrees of domination was observed between Magura Cave and La Garma, Spain (Schabereiter-Gurtner

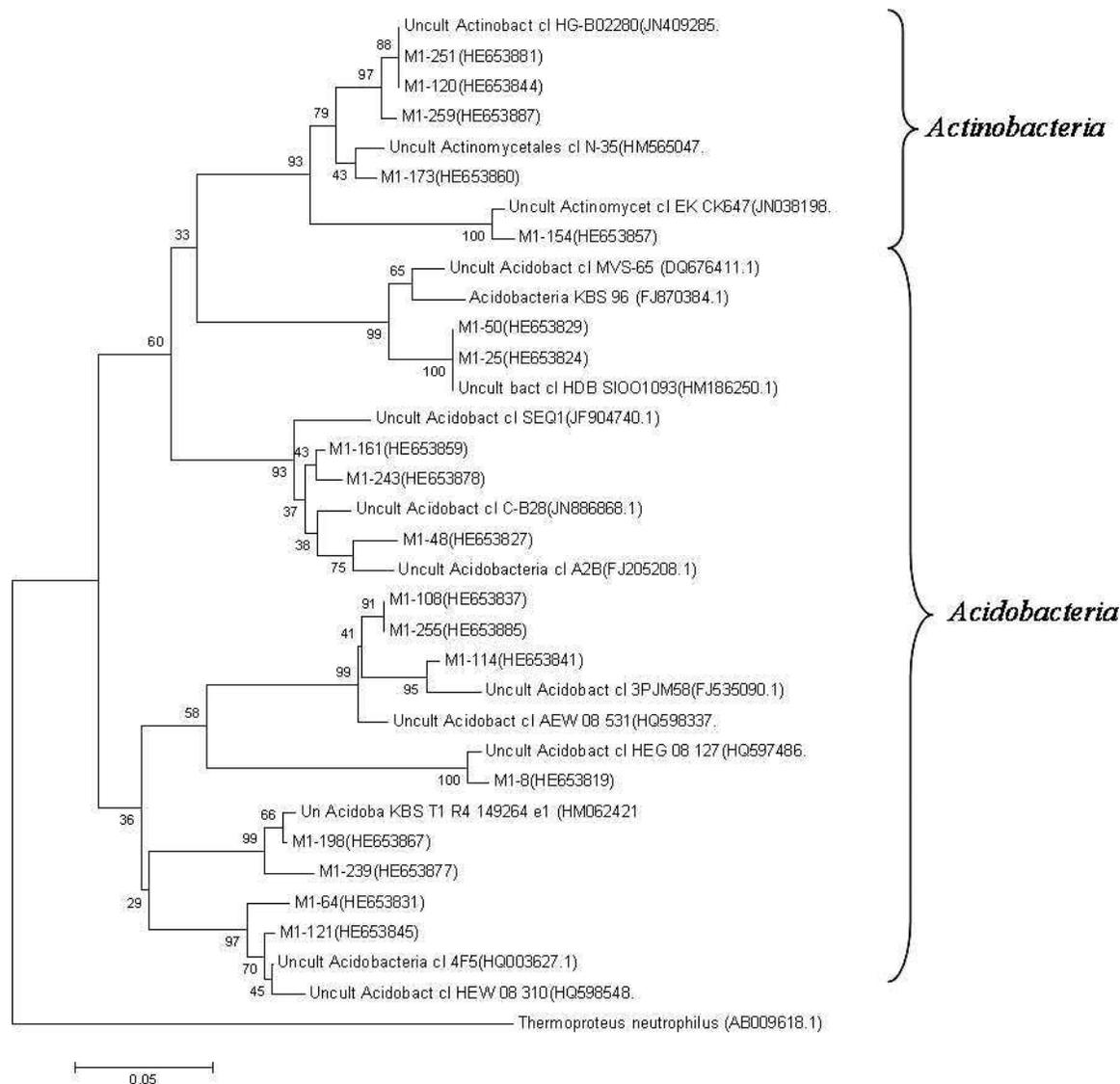


Figure 4. Neighbor-joining tree, calculated using evolutionary distances, based on *Acidobacteria* and *Actinobacteria* 16S rRNA sequence types from Magura Cave. Scale bar 5% substitutions in nucleotide sequence. Bootstrap values are shown at branching points (percentage of 1000 resamplings). Sequence accession numbers are given in parenthesis. *Thermoproteus neutrophilus* was used as the out-group.

et al., 2004). However, *Actinobacteria* was among the dominant groups and *Nitrospirae* was weakly represented in La Garma.

Nitrospirae is not a phylum established in all caves. It was found to be a dominant group only in Magura Cave. It was reported as weakly represented in Llonín and La Garma (Schabereiter-Gurtner et al., 2004) and was absent in Altamira and Tito Bustillo. Although a small number of culturable *Nitrospirae* are known, all they are obligate chemolithotrophs, receiving energy for their growth from nitrite oxidation.

The degree of *Acidobacteria* domination is similar in Magura, Altamira (Schabereiter-Gurtner et al., 2002a),

Tito Bustillo (Schabereiter-Gurtner et al., 2002b), and La Garma (Schabereiter-Gurtner et al., 2004), but this phylum was absent in Llonín. Despite the small number of isolated culturable representatives of the phylum *Acidobacteria*, related sequences have been identified from various environments, such as soil, fresh water sediment, and sewage sludge (Tringe et al., 2005; Janssen, 2006). Initially, low pH was accepted as an obligate condition for their growth (Kuske et al., 1997), but 16S rRNA genes related to *Acidobacteria* have been isolated from neutral soils (Dunbar et al., 1999). Quaiser et al. (2003) suggested including non-acidophilic microorganisms as a separate group in the phylum. As this phylum was recently

Table 1. Phylogenetic affinity of 16S rRNA gene sequences of bacteria identified in the Magura Cave to sequences from the phyla *Planctomycetes*, *Firmicutes*, *Chloroflexi*, and *Gemmatimonadetes*. Sequences with similarity to the closest neighbor less than 97% are noted in bold.

Clone and sequence	Closest identified phylogenetic relatives	Phylum	Fragment length (bp)	Clone number
M1-146 (HE653854)	Uncultured bacterium clone FOOS7B_88 - 95%	<i>Planctomycetes</i>	1476	1
M1-117 (HE653843)	Uncultured Planctomycetes bacterium - 97%		972	1
M1-237 (HE653876)	Uncultured Gemmatimonadetes bacterium clone g55 - 99%	<i>Gemmatimonadetes</i>	1065	2
M1-26 (HE653825)	Uncultured Gram-positive bacterium clone FTL22 - 97%	<i>Firmicutes</i>	1420	1
M1-204 (HE653870)	Uncultured bacterium clone SWB36 - 98%		1412	1
M1-144 (HE653852)	Uncultured Chloroflexi bacterium clone II10F - 92%	<i>Chloroflexi</i>	1393	1
M1-145 (HE653853)	Uncultured Gram-positive bacterium clone FTL22 - 97%		1458	1
M1-181 (HE653862)	Uncultured bacterium clone HDB_SIPP610 - 99%		1401	1

discovered, their ecology and metabolism are not yet sufficiently clear.

A high degree of representation of the phylum *Actinobacteria* was reported for the Paleolithic Cave of Bats, Zuheros, Spain (Urzi et al., 2010). As typical heterotrophs, *Actinobacteria* actively participate in the

carbon cycle by degradation of organic wastes. Although Groth and Saiz-Jimenez (1999) described *Actinomycetes* as a dominant group in caves due to low temperature and high redox potential, this group was poorly presented in the Magura Cave. Despite the fact that *Streptomyces* representatives are commonly accepted as obligate inhabitants

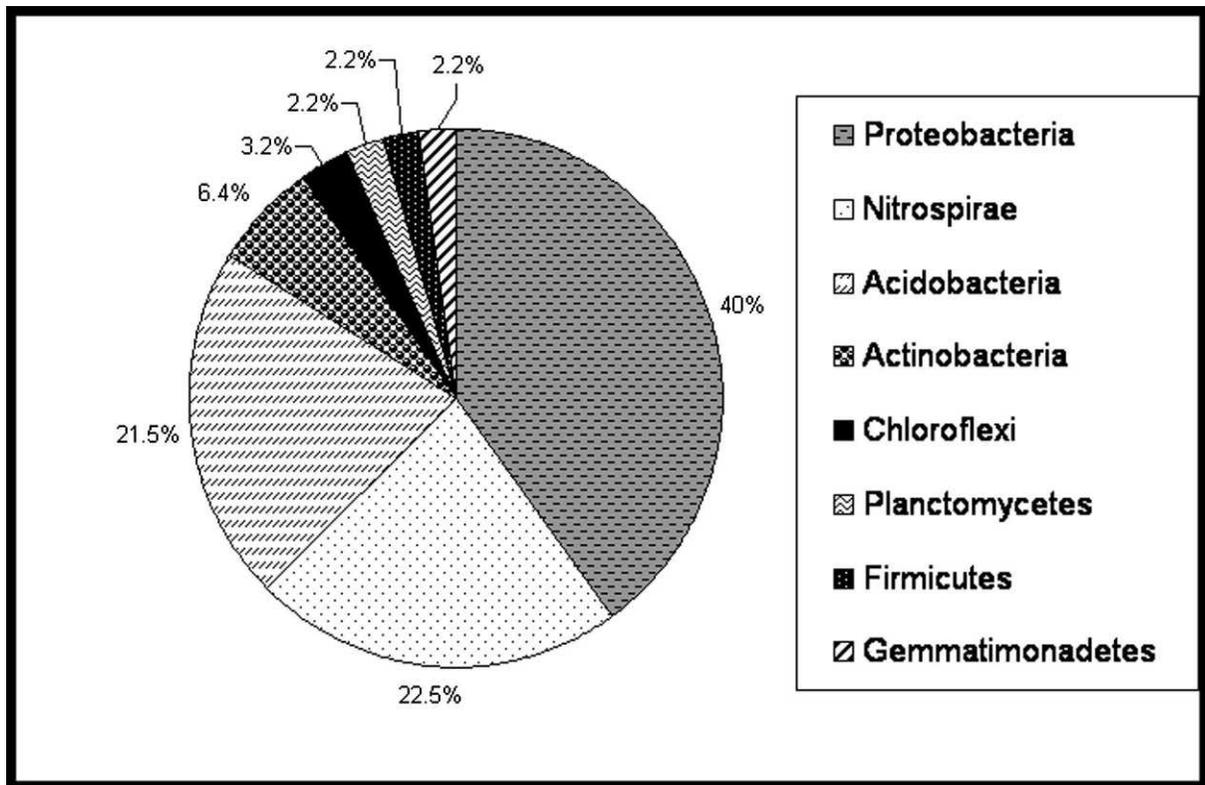


Figure 5. Comparative representation of the bacterial phyla in the sample from the area of the prehistoric paintings in Magura Cave.

Table 2. Represented percentages of bacterial phyla in several European caves with prehistoric paintings.

Represented groups, %	Magura Cave, Bulgaria ^a	La Garma, Spain ^b	Llonín, Spain ^b	Altamira, Spain ^c	Tito Bustillo, Spain ^d
<i>Proteobacteria</i>	39.8	32.7	59.2	52.3	48.8
<i>Nitrospirae</i>	22.5	3.4	3.7	0	0
<i>Acidobacteria</i>	21.5	24.1	0	23.8	29.2
<i>Actinobacteria</i>	6.4	19.0	22.2	4.8	9.8
<i>Planctomycetes</i>	2.2	0	0	4.8	2.4
<i>Firmicutes</i>	2.2	13.8	3.7	0	0
<i>Chloroflexi</i>	3.2	1.7	0	4.8	7.3
<i>Gemmatimonadetes</i>	2.2	0	0	0	0
<i>Cytophaga-Flexibacter-Bacteroides</i>	0	3.4	11.1	9.5	2.4

^a This study.^b Schabereiter-Gurtner et al., 2004.^c Schabereiter-Gurtner et al., 2002a.^d Schabereiter-Gurtner et al., 2002b.

of caves, especially near the entrance (Urzi et al., 2002), their absence in Magura Cave's Gallery could be explained by its deep position.

Magura Cave is the only one among the compared caves in which representatives of the phylum *Gemmatimonadetes* were identified. Isolated clones related with the phylum *Gemmatimonadetes* grouped with unculturable clones. Despite the fact that only one culturable representative, *Gemmatimonas aurantiaca* gen. nov., sp. nov has been isolated (Zhang et al., 2003), identification of sequences from this phylum from different niches suggests that it is widely distributed in nature.

Some authors suggest that the anthropogenic factor has little or no influence on bacterial communities (Laiz et al., 2000; Schabereiter-Gurtner et al., 2004). However, other authors accept identification of some bacteria, such as *Bacillus*, *E. coli*, and *Staphylococcus aureus*, as indicators for human presence (Lavoie and Northup, 2006). Ikner et al. (2007) suggested that *Proteobacteria* is slightly represented in highly visited caves (more than 200,000 visitors per year) instead of *Firmicutes*. However, a low frequency for the phylum *Firmicutes* was observed not only for Magura and Llonín; it was totally absent in Altamira and Tito Bustillo, two caves intensively visited in the past. Culturable representatives of this phylum were isolated from an area without paintings in Altamira (Canaveras et al., 2001). A low number or absence of *Firmicutes* sequences in cave communities suggest that this phylum does not play a key role for the functioning of cave communities. A sequence related with *Enterobacter* (HE653855) was isolated from Magura Cave. Identification of pathogens could be attributed not only to visitors but also to bats and insects inhabiting the caves (Jurado et al., 2010). People could also change structure of the community by their actions, for example the major changes

of microbial structure in the cave Lascaux, France, where *Acidobacteria*, *Actinobacteria*, and *Nitrospira* were replaced by *Beta* and *Gamma Proteobacteria* after use of fungicides to prevent fungi invasion (Dupont et al., 2007).

Together with the high number of phylogenetic groups represented in Magura Cave, the current investigation revealed a presence of sequences suggestive of unknown bacterial taxa. Novelty of sequences is usually discussed in three levels of distance: 3%, 5% and 15%, corresponding to thresholds for novelty on the levels of species, genus, and phylum respectively (Hugenholtz et al., 1998; Pašić et al., 2010). The degree of novelty of the sequences from Magura Cave deposited in GenBank was up to 12% phylogenetic distance (sequence HE653833). We didn't establish phylogenetic types having < 85% identity to the known sequences, suggesting that all sequences could be affiliated with known phyla. More than 30.9% (21 clones) showed < 97% similarity with the sequences deposited in GenBank; similarity was less than 95% for thirteen of them, suggesting existence of novel taxa at the genus level in Magura Cave. Novel phylotypes were identified in six out of the eight phyla presented in the cave. New sequences represented half of the identified sequences in two of the phyla, *Nitrospira* and *Acidobacteria*. Two of *Nitrospira* sequences showed more than 10% divergence. Some of the new sequences were represented only once in the clone collection; but others like M1-48 (*Acidobacteria*) and M1-139 (*Nitrospirae*) occurred in four and three clones, respectively, proving a comparatively high abundance of these new lineages in the cave.

Lack of enough nutrients in caves determines metabolic flexibility of bacterial communities. The bacterial community in Magura Cave comprises bacteria with different metabolisms, both autotrophic and heterotrophic. The Gallery is dark, and we expected lithotrophic microorganisms,

rather than phototrophic ones, to be the primary source of organic productivity. Bacteria participating in oxidation of sulfur, sulfide, manganese, iron, nitrite, and ammonium ions have been identified in several caves (Northup et al., 2000; Vlasceanu et al., 2000; Engel et al., 2001; Holmes et al., 2001) and represent an important component of the bacterial cave community. The bacteria from phylum *Nitrospirae* and the genus *Nitrococcus* in the *Gamma Proteobacteria* identified in Magura Cave oxidize nitrites to nitrates. Sulfur bacteria are considered a main group participating in karst solubility and formation of secondary cave structures. Sulfur bacteria from the family *Chromatiaceae* were detected in Magura Cave. Other identified participants in the sulfur cycle were sulfate-reducing bacteria from the order *Syntrophobacterales*. The well-established chemolithotrophic part of the community provides nutrients to support chemoorganotrophic growth. Organotrophs affiliated with *Alpha* and *Beta Proteobacteria*, *Planctomycetes*, and *Firmicutes* were identified in Magura Cave. Interpretation of this information, however, should be very careful, as sometimes related organisms can express different physiologies; moreover, the same group can express different phenotypic properties in different niches (for example *Actinomycetales* and *Actinobacteria*).

Although Barton and Northup (2007) suggest that very few microbial species in caves are capable of assimilating limited but chemically complex nutrients, our phylogenetic analysis of the bacterial diversity in Magura Cave revealed that its inhabitants are phylogenetically diverse, with lineages referred to a number of different bacterial phyla and to different metabolic types.

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