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Diversity and biosynthetic potential of culturable aerobic heterotrophic bacteria isolated from Magura Cave, Bulgaria

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Abstract: Biocapacity of bacteria inhabiting karstic caves to produce valuable biologically active compounds is still slightly investigated. A total of 46 culturable heterotrophic bacteria were isolated under aerobic conditions from the Gallery with pre-historical drawings in Magura Cave, Bulgaria. Phylogenetic analysis revealed that most of bacterial isolates affiliated with *Proteobacteria* (63%), followed by *Actinobacteria* (10.9%), *Bacteroidetes* (10.9%), and *Firmicutes* (6.5%). A strong domination of Gram-negative bacteria (total 81%) belonging to nine genera: *Serratia*, *Pseudomonas*, *Enterobacter*, *Sphingobacterium*, *Stenotrophomonas*, *Commamonas*, *Acinetobacter*, *Obesumbacterium*, and *Myroides*, was observed. Gram-positive isolates were represented by the genera *Bacillus*, *Arthrobacter*, and *Micrococcus*. One isolate showed a significant phylogenetic distance to the closest neighbor and could represent a novel species. Heterotrophic bacterial isolates from Magura Cave were investigated for hydrolytic enzymes production, antimicrobial and hemolytic activity. Predominance of producers of protease (87%), followed by xanthan lyase (64%), lipase (40%), β -glycosidase (40%), and phytase (21%) was observed. Over 75% of the isolates demonstrated antimicrobial and hemolytic activity. The results suggest that heterotrophic bacteria isolated from Magura Cave could be a valuable source of industrially relevant psychrotolerant enzymes and bioactive metabolites. This study is a first report on the taxonomic composition and biological activity of culturable bacteria inhabiting a cave in Bulgaria.

Keywords: caves; Magura Cave; culturable bacteria; 16S rDNA; enzyme production; antimicrobial activity; hemolytic activity

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INTRODUCTION

Caves are nutrient-poor, dark ecosystems with relatively constant low temperature and high relative humidity in the dark zone deep within the cave. Although providing extreme conditions for life, the caves are colonized by diverse psychrophilic (cold-loving) and psychrotolerant (cold-adapted) microorganisms that are able to grow in these environments (Schabereiter-Gurtner et al., 2002a; Barton & Northup, 2007). Processes of disintegration and formation of cave saltpeter, mineral oxides accumulations, coloration, and moonmilk deposits in many karstic caves have been linked with microbial activity (Castanier et al., 2000).

At present, most of the well-known and visited caves containing valuable pre-historical paintings are affected by progressive microbial colonization

and biodeterioration, and the control, treatment, and preservation of these specific environments is a serious worldwide problem (Cañaveras et al., 2001; Allemand & Bahn, 2005; Fox, 2008). Today, most caves containing such paintings are closed off to visitors as a conservative measure to prevent further degradation of valuable cultural heritage sites. The literature on microbial communities in subterranean environments is mainly restricted to caves found in Spain, Italy, France, Romania, and the USA. A long-term microbiological study of the cave microflora is being conducted in Altamira Cave (Spain) and Lascaux Cave (France), both containing Paleolithic paintings (Schabereiter-Gurtner et al., 2002a; Bastian et al., 2009).

Despite their archaeological importance, our knowledge on the microbiology of caves is still scanty and incomplete. Although geomicrobiological environmental investigations are widely presented, a

significant increase in biospeleological research was observed within the last two decades (Urzi et al., 2010). Caves can be considered extreme environments for life providing ecological niches for highly specialized microorganisms (Schabereiter-Gurtner et al., 2002b). These microorganisms, called extremophiles, produce enzymes that are functional under extreme conditions (Van den Burg, 2003). Although many papers have been published on isolation of psychrophilic and psychrotolerant bacteria from cold environments and their enzymatic activities, caves are still under-explored as an object for such investigations. Our knowledge of cave microbial diversity is limited; therefore, great potential exists to find novel microorganisms in caves (Engel, 2010). One of the essential mechanisms for adaptation of microorganisms to cold environments is the activity of their enzymes at low temperatures. In recent years, there is an increased interest in cold-adapted microorganisms and their enzymes because of their great biotechnological potential, offering numerous economic and ecological advantages. Because of the unique properties, cold-adapted enzymes could be extremely useful in different biotechnological fields, for example, in the detergent and food industry, biotransformation, environmental bioremediation, molecular biology, diagnostics, biosensors and bioreporters (Russell, 1998; Gerday et al., 2000; Cabeza et al., 2011; Cavicchioli et al., 2011).

Karstic Magura Cave is situated near the village of Rabisha, North-West Bulgaria. Being a monument of culture and a tourist destination, it is one of the biggest and the most beautiful caves in Bulgaria. In one of the Galleries unique prehistorical drawings were discovered that represent carvings into the stone filled with bat guano (Fig. 1). Ancient drawings are multi-layered, dating back from the Late Neolithic and Eneolithic Ages, to the the early Bronze Age, and are unique for the Balkan Peninsula (Stoytchev, 1994; 1995). They represent different religious events and feasts in separate groups. The Solar calendar from the Late Neolith is the earliest solar calendar discovered in Europe; it depicts five festivals and 366 days and may have represented a shrine for the prehistoric settlement. As a result, Magura Cave was placed on the tentative list for consideration as a World Heritage Site by UNESCO in 1984. The cave has been opened for visitors only for three years; however, this resulted in changes in cave's microclimate due mainly to the presence of artificial light and emitted heat, and in enhanced deterioration of many of the drawings by vandal effects. That is why, in 2008 the so called Gallery with the drawing was closed to the public as a conservative measure to preserve the valuable drawings.

No studies have been undertaken until now for isolation and cultivation of microorganisms inhabiting Bulgarian caves and investigation of their biosynthetic capacity. In this study, culturable aerobic heterotrophic bacteria inhabiting the Gallery with the drawings in Magura Cave were isolated and characterized, and their biosynthetic abilities were investigated in aim to identify promising psychrotolerant bacterial strains, producers of industrially relevant enzymes and antimicrobial compounds.

Fig. 1. Rock wall drawings in the gallery with the drawings in Magura Cave.

MATERIALS AND METHODS

Sampling site

Geological analysis showed that the formation of Magura Cave has started 15 million years ago on a calcareous hill (at 461 m above the sea level) composed of a succession of subhorizontal decimeter-thickbeds of Cretaceous calcarenitic limestones. Magura Cave consists of a main gallery and three side branches with a current overall length of approximately 3,000 m (http://www.magura-cave.com/1024x768/eng/the_cave.htm). The northern end of "the Landslip" is connected to the alley with the drawings. On its main axis, the corridor measures 240 m in length and covers 3,750 m² in

area. Its maximum height is 24 m. The unique cave drawings are situated in this corridor. The isolated location of the Gallery with the drawings and its longness contribute to the maintenance of a relatively constant temperature of around 12 °C and relative humidity above 90%.

Sample collection

Samples were collected in the beginning of April 2011 from the Gallery with the drawings in Magura Cave. Samples of about 30 to 50 g each were collected in sterile glass jars by gently scrapping by sterile scalpels of rock walls (in depth up to 1 mm) in the vicinity of separated drawings at a level 30-50 cm above the floor. A control sample was also collected from rock wall surfaces of a side area of the Gallery without drawings. In parallel, wall samples, sediment samples from the floor (containing bat guano), and water samples were also collected by the research team from Sofia University. Collected samples were transferred to the laboratory in a thermostat bag, kept in a refrigerator for about 18 h and used as inocula for enrichment.

Isolation and growth of heterotrophic bacteria

Enrichment for isolation of bacterial cultures was performed for each sample (about 10 g) aerobically at 14 °C during 10 days including two transfers, in 100-ml Erlenmeyer flasks containing 10-ml sterile nutrient broth (NB). After two transfers, a loopful of each inoculum source was streaked onto nutrient agar (NA) plates. The plates were then incubated at 14 °C and monitored at 24 h intervals for a period of a week in order to isolate a variety of growing bacteria. Different types of colonies were picked after incubation and streaked onto NA plates to obtain pure cultures. All isolates were maintained by monthly transfers onto slants containing NA and stored at 4 °C. The strains were stored also at -80 °C in NB supplemented with 30% (v/v) glycerol.

Growth of bacteria isolated from Magura Cave was followed at temperatures of 4 °C, 10 °C, 20 °C, 28 °C, and 40 °C. Isolates were cultivated at the indicated temperatures in test-tubes containing NB with added aliquots of each bacterial inoculum, and the growth was monitored until the stationary phase by measuring the optical density at 570 nm (OD_{570}).

16S rDNA amplification and restriction analysis

The isolates were subjected to 16S rDNA analysis to determine their phylogenetic affiliation. Genomic DNA from a broth culture of the strains was isolated using GenElute Bacterial Genomic DNA kit (Sigma). The small-subunit rRNA gene was amplified from the extracted DNA using universal bacterial primers specific to 16S rRNA gene, primer 8F and primer 1492R (Weisburg et al., 1991). The PCR reaction mixture (20 µl) contained: 1 to 10 ng of template, Prime Taq Premix 2x (GENET BIO) containing 1 U/10 µl DNA Polymerase, 20 mM Tris-HCl, 80 mM KCl, 4 mM $MgCl_2$, enzyme stabilizer, sample, loading dye, pH 9.0, 0.5 mM of each dATP, dCTP, dGTP, dTTP, and 400 nM of each primer. The reaction mixture

was incubated in a T100 Thermal Cycler (BIO-RAD) for an initial denaturation at 94 °C for 5 min followed by 30 cycles of 94 °C for 30 s, 55 °C for 30 s, and 72 °C for 60 s, then a final extension step at 72 °C for 5 min. The amplified 16S rRNA genes of the isolates were subjected to two separate RFLP analyses, using four base pairs restriction enzymes in order to maximize the RFLP resolution. Seven µl of the amplified PCR products were separately digested with 5 U of restriction endonucleases *MspI* and *HaeIII* (Fermentas) in a final volume of 10 µl for 2 h at 37 °C. Isolates with identical RFLP profiles were considered to be phylogenetically identical. Representatives of each RFLP pattern group were chosen for sequencing (total 24 strains).

The full sequence (8-1,492 nt; *E. coli* numbering) of the 16S rRNA gene of the strains was determined with Applied Biosystems model 373A DNA sequencer by using the ABI PRISM cycle sequencing kit (Macrogen, South Korea). DNA sequence analyses were performed using BLASTn (Altschul et al., 1990) and Ribosomal Database Project resources (Maidak et al., 1994) to determine their close relatives and approximate phylogenetic affiliations. Multiple sequence alignments were obtained using CLUSTALX (Thompson et al., 1997). The phylogenetic analysis was performed using the program PAUP* Version 4.0b10 (Swofford, 2002). The sequence that showed more than 3% divergence with the closest phylogenetic neighbor was submitted to a database under accession number HE663456. All other sequences were not deposited due to the lack of novelty and were used for phylogenetic identification of the isolates.

Screening for enzyme and hemolytic activities

Hydrolytic enzyme production by bacterial isolates from Magura Cave was determined qualitatively by the agar diffusion method. Petri plates contained basal mineral medium with 1.5% agar and a substrate specific for the corresponding activity. Basal mineral medium (pH 7.0) contained (%): KH_2PO_4 0.1, $(NH_4)_2SO_4$ 0.5, $MgSO_4 \cdot 7H_2O$ 0.01, and NaCl 0.01. A loop of each of 24 h pure bacterial culture grown on NA was spread via a needle on the test media. The plates were then incubated in the dark at 28 ± 2 °C for 4 to 10 days depending on the activity tested. Two replicates were carried out for each sample. The cultures that showed growth, clearing zones, or color-diffusion zones on respective specific media were considered as positive cultures. Extracellular protease production was determined by using 30% (v/v) skim-milk or 1% (w/v) gelatin; a clear zone around the colony indicated presence of proteolytic activity (PrA). The α -amylase activity (AmA) was determined by using 1% (w/v) insoluble starch and detected as a clear zone around the colony (Dhawale et al., 1982). Ribonuclease (RNase) or desoxiribonuclease (DNase) activities were detected in test agars containing 0.5% (w/v) RNA or DNA, respectively. Growth with RNase/DNase production appeared surrounded by a clear halo as a result of the degradation of RNA/DNA to soluble nucleotides, which were not precipitated by the added 1N hydrochloric acid. Lipolytic activity (LipA)

was tested by hydrolysis of 1% Tween 80 in a basal agar medium amended with 0.01% $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$ and was seen as either a visible precipitate of the calcium chloride-lipid complex around a colony, or as a clearing zone around a colony due to complete degradation of the salt complex (Smilbert, 1994). The strains were screened for cellulase activity in agar medium with 2% (w/v) CMC (Carboxymethyl cellulose) as a substrate. The plates were incubated and stained with Congo red dye and destained (Teather & Wood, 1982). The positive cellulase activity was shown as a presence of yellow halo against red background. Urease activity was tested on urea agar plates with 2% urea and phenol red and detected as a formation of a pink-colored clear zone around the colonies (MacFaddin, 2000). Phytase activity (PhyA) was determined after growth of the strains up to 10 days on agar plates containing phosphate-depleted basal medium supplemented with 0.5% (w/v) sodium phytate (Sigma, USA) as a substrate following procedure described by Bae et al. (1999). The β -glucosidase activity (β -gluA) was evaluated on a selective medium containing 0.4% arbutin (hydroquinone β -D-glucopyranoside) (Santa Cruz, USA); a positive result was indicated by the growth and dark brown halo around the colonies (Mendes Ferreira et al., 2001). Polygalacturonase (PGAse) activity was detected by the degradation of polygalacturonic acid using Ruthenium red plate assay (Cotty et al., 1990). Xanthan lyase activity was detected by the growth of the strains in liquid basal mineral medium containing 0.2% (w/v) xanthan as a sole carbon source.

Extracellular hemolytic activity was tested by dropping of 0.1 ml cell suspensions of each bacterial culture (grown on NB for 20-h) into wells on blood agar plates. After incubation for 48 h to 72 h at $28 \pm 2^\circ\text{C}$, the zones of hemolysis of erythrocytes were measured.

Screening for antimicrobial activity

Bacterial isolates from Magura Cave were tested for their ability to inhibit the growth of four indicator cultures: *Bacillus subtilis* ATCC 6633, *Pseudomonas aeruginosa* NBIMCC 1390 (National Bank of Industrial Microorganisms and Cell Cultures, Bulgaria; <http://www.nbimcc.org/en/about.htm>), the phytopathogenic bacterial strain *Xanthomonas oryzae*, causing bacterial blight of rice, and the yeast *Rhodotorula mucilaginosa* 6526 (formerly *Rhodotorula rubra*). Cave bacterial isolates were grown on NB for 20 h (in exponential growth phase) before detection of an antimicrobial activity by the agar well diffusion method. Indicator cultures were spread on the agar media, followed by hole-punches (6-mm in diameter) that were filled with 0.1 ml cell suspension of each cave organism. After overnight incubation at $28 \pm 2^\circ\text{C}$, clear zones (halos) were recorded as a zone diameter (in millimeters) around the wells minus 6-mm diameter of the well.

RESULTS

Isolation and identification of culturable bacteria from Magura Cave

A total of 46 aerobic heterotrophic bacterial strains were isolated from the Gallery with the drawings in

Magura Cave (Table 1). Fourteen bacterial strains were recovered from the painted surfaces, 25 from adjacent rock wall surfaces, 5 from bat guano (in sediment samples), and 2 from water samples. Strains No 1-25 were isolated in Faculty of Biology at Sofia University; other strains were isolated in the Institute of Microbiology-BAS and marked shortly as IM. After amplification and restriction of the 16S rRNA gene for each isolate their restriction profiles were compared and the strains were distributed into eighteen groups. Representatives of four phylogenetic groups: *Proteobacteria*, *Actinobacteria*, *Firmicutes*, and *Bacteroidetes* were identified with a strong *Proteobacteria* domination (Fig. 2). After sequencing and phylogenetic analysis, a strong domination of Gram-negative heterotrophic bacteria was established (81%), belonging to nine genera: *Serratia*, *Pseudomonas*, *Enterobacter*, *Sphingobacterium*, *Stenotrophomonas*, *Commamonas*, *Acinetobacter*, *Obesumbacterium*, and *Myroides*, with predominance of *Serratia* and *Pseudomonas* strains. Gram-positive isolates (19%) were representatives of three genera: *Bacillus*, *Arthrobacter*, and *Micrococcus*. Isolate IM13 was determined to share 94% sequence similarity of its 16S rRNA gene to the nearest phylogenetic relative of the genus *Myroides* and may represent a novel species.

Growth patterns of bacterial isolates

Growth of the bacterial isolates from Magura Cave was followed at temperatures of 4°C , 10°C , 20°C , 28°C , and 40°C , and values of OD_{570} in the beginning of stationary phase were compared. The growth of Magura Cave isolates at different temperatures is shown in Table 1 and Fig. 3. The majority of isolates (33 strains) were psychrotolerant determined by their ability to grow at both 4°C (although too slow) and above 20°C , highest growth rate observed at temperatures between 20 and 30°C , and may have upper limits as high as 40°C (Morita, 1975). Thirteen strains appear to be mesophilic, for which the growth at 40°C was from 2.5 to 9 times higher (depending on the strain) than the growth determined at 4°C .

Hydrolytic enzymes and hemolytic activity

Heterotrophic bacteria isolated from Magura Cave were tested for hydrolytic enzymes activity on

Fig. 2. Phyla distribution of heterotrophic bacteria isolated from the Gallery with the drawings in Magura Cave.

Table 1. Properties of heterotrophic bacteria recovered from the Gallery with the drawings in Magura Cave.

Isolate code*	Gram staining	Sample type	Growth at 4 °C (OD ₅₇₀)	Growth at 40 °C (OD ₅₇₀)	Nearest relative on the basis of 16S rDNA sequences similarity
B1	Gram negative	Guano	0.078	0.037	<i>Enterobacter</i> sp. 2B1C (EU693561), 99%
B2	Gram negative	Guano	0.389	0.118	<i>Pseudomonas fragi</i> (AB685688), 99%
B3 (B8, G7)	Gram negative	Drawings	0.205	0.123	<i>Pseudomonas fluorescens</i> 15834 (AB680973), 99%
B4	Gram negative	Drawings	0.127	0.309	ND
B5	Gram negative	Drawings	0.126	0.338	<i>Serratia</i> sp. a101-87 (HM468088), 100%
B6 (B11)	Gram negative	Guano	0.088	0.327	<i>Enterobacter asburiae</i> JCM6051 (NR_024640), 99%
B7	Gram negative	Guano	0.190	0.085	<i>Sphingobacterium</i> sp. Ag8 (JN257086), 99%
B8	Gram negative	Water	0.160	0.157	<i>P. fluorescens</i> 15834 (AB680973), 99%
B9	Gram positive	Rock wall	0.087	0.029	<i>Bacillus safensis</i> KTT-25 (HQ696447), 98%
B11	Gram negative	Drawings	0.055	0.327	<i>Enterobacter asburiae</i> JCM6051 (NR_024640), 99%
B13	Gram negative	Drawings	0.197	0.089	<i>Sphingobacterium</i> sp. Ag8 (JN257086), 99%
B14	Gram negative	Drawings	0.342	0.227	<i>Serratia</i> sp. L0305 (JN169125), 99%
B16	Gram negative	Rock wall	0.056	0.013	<i>Serratia</i> sp. a101-87 (HM468088), 100%
B17	Gram positive	Drawings	0.363	0.170	<i>Micrococcus luteus</i> CJ-G-TSA7 (HM584259), 99%
B18	Gram negative	Water	0.072	0.435	<i>Serratia</i> sp. a101-87 (HM468088), 100%
B19	Gram negative	Drawings	0.029	0.078	ND
B20	Gram negative	Drawings	0.057	0.312	<i>Serratia</i> sp. a101-87 (HM468088), 100%
CB9	Gram positive	Drawings	0.035	0.253	<i>M. luteus</i> CUB12 (JF802083), 99%
CB11	Gram negative	Guano	0.077	0.322	<i>Serratia</i> sp. a101-87 (HM468088), 100%
CB12	Gram positive	Drawings	0.029	0.271	<i>Bacillus flexus</i> SL21 (JN645852), 99%
CB13	Gram negative	Drawings	0.175	0.294	ND
CB15 (B7, B13, IM3)	Gram negative	Drawings	0.029	0.014	<i>Sphingobacterium</i> sp. Ag8 (JN257086), 99%
CB16	Gram positive	Drawings	0.023	0.185	<i>Bacillus amyloliquefaciens</i> ZP0308 (JN021517), 99%
G5	Gram negative	Rock wall	0.153	0.274	<i>Acinetobacter</i> sp. CJ-S-PYD4 (HM584281), 100%
G7	Gram negative	Rock wall	0.150	0.040	<i>P. fluorescens</i> 15834 (AB680973), 99%
IM1 (IM2, IM4, IM16)	Gram negative	Rock wall	0.322	0	<i>Serratia</i> sp. 136-2 (EU557341), 99%
IM2	Gram negative	Rock wall	0.222	0.174	<i>Serratia</i> sp. 136-2 (EU557341), 99%
IM3	Gram negative	Rock wall	0.261	0.189	<i>Sphingobacterium</i> sp. Ag8 (JN257086), 99%
IM4	Gram negative	Rock wall	0.514	0	<i>Serratia</i> sp. 136-2 (EU557341), 99%
IM5	Gram negative	Rock wall	0.347	0	<i>P. fluorescens</i> LMG 14576 (GU198121), 100%
IM6	Gram positive	Rock wall	0.037	0	<i>Arthrobacter</i> sp. R4 (FN392695), 99%
IM7	Gram negative	Rock wall	0.246	0.206	<i>Obesumbacterium proteus</i> 511 (FJ492810), 99%
IM8	Gram negative	Rock wall	0.403	0	<i>Serratia proteamaculans</i> DSM4543 (NR_025341), 99%
IM9	Gram negative	Rock wall	0.288	0	ND
IM10 (IM11)	Gram positive	Rock wall	0.312	0	<i>Arthrobacter</i> sp. R-36193 (FR682676), 99%
IM11	Gram positive	Rock wall	0.150	0.183	<i>Arthrobacter</i> sp. R-36193 (FR682676), 99%
IM12 (IM5, IM21)	Gram negative	Rock wall	0.400	0.172	<i>P. fluorescens</i> LMG 14576 (GU198121), 100%
IM13	Gram negative	Rock wall	0.155	0.010	<i>Myroides</i> sp. IT-2012 (HE663456), 94%
IM14 (B5, B16, B18, B20, CB11)	Gram negative	Rock wall	0.335	0	<i>Serratia</i> sp. a101-87 (HM468088), 100%
IM15	Gram negative	Rock wall	0.305	0	<i>Pseudomonas putida</i> (AB681704), 99%
IM16	Gram negative	Rock wall	0.321	0.131	<i>Serratia</i> sp. 136-2 (EU557341), 99%
IM17 (IM19)	Gram negative	Rock wall	0.042	0	<i>Stenotrophomonas</i> sp. DIC6JA (FJ668834), 99%
IM18	Gram negative	Rock wall	0.244	0.025	<i>Comamonas</i> sp. BM-9_6 (AY635871), 99%
IM19	Gram negative	Rock wall	0.053	0.023	<i>Stenotrophomonas</i> sp. DIC6JA (FJ668834), 99%
IM20	Gram negative	Rock wall	0.295	0.024	<i>Pseudomonas plecoglossicida</i> (AY972231), 100%
IM21	Gram negative	Rock wall	0.139	0	<i>P. fluorescens</i> LMG 14576 (GU198121), 100%

ND, not determined.

*, strains in the brackets were referred to the same restriction group and were non-sequenced.

Fig. 3. Growth of bacterial isolates (IM1-21) from Magura Cave at different temperatures

agar medium using specific substrates. Isolates were screened for production of the main hydrolytic enzymes (proteases, lipases, amylases, nucleases) as well as for production of some enzymes with potential biotechnological application, such as phytase, xanthan lyase, polygalacturonase, and β -glucosidase. We found that the most often observed hydrolytic enzymes were proteases and xanthan lyases than the other enzymes (Table 2). Up to 87% of the isolates showed proteolytic activity, and skim milk appeared to be more favorable substrate than gelatin (68% and 51% of isolates, respectively). About 64% of isolates were able to grow on xanthan mineral medium indicating presence of xanthan lyase activity; 40% of isolates showed lipase and β -glycosidase activity, and 21% – phytase activity. Polygalacturonic acid was hydrolyzed only by two strains, IM14 and IM15. RNase and amylase activity were detected only for a strain CB16. No hydrolysis of DNA and CMC was observed.

It was found that about 76% of the cave isolates exhibited hemolytic activity after testing on blood agar plates (Table 3). Among them, 40% showed weak hemolytic zone up to 1.5 mm; 15% showed zone of 2-2.5 mm, and 21% of the strains – zone of 3-4 mm.

Antimicrobial activity

It was established that about 50% of the isolates from Magura Cave exhibited inhibitory activity against *P. aeruginosa* and the yeast strain *R. mucilaginosa* (Table 3). A significant inhibition zone of 20-mm was observed for isolate B3 (*Pseudomonas fluorescens*) against *P. aeruginosa*, and 16-mm against *R. mucilaginosa*. Isolates IM15 (*Pseudomonas* sp.), IM16 (*Serratia* sp.), IM18 (*Comamonas* sp.), and IM20 (*Pseudomonas plecoglossicida*) inhibited significantly the growth of *B. subtilis*. About 15% of the strains exhibited antimicrobial activity against *X. oryzae*; the highest inhibition was achieved by isolates B7 (*Sphingobacterium* sp.), IM18 (*Comamonas* sp.), and IM20 (*P. plecoglossicida*) with a zone of inhibition of 10-mm in diameter. Among isolates, IM16 (*Serratia* sp.), IM18 (*Comamonas* sp.), and IM20 (*P. plecoglossicida*) could be considered as the most active inhibiting the growth of all tested cultures.

DISCUSSION

This study demonstrates that surfaces of drawings and rock walls in the Gallery with the drawings in

the karstic Magura Cave are inhabited by diverse heterotrophic bacteria. By using NA, we isolated 46 culturable aerobic heterotrophic bacteria referred to four phyla. The relatively low number of isolated bacteria could be due to using nutrient-rich media, which may restrict the growth of bacteria which may be better adapted to the oligotrophic conditions of the cave environment. The observed domination of *Proteobacteria* is in a good agreement with previous studies in Kartchner Caverns, Arizona (Ikner et al., 2007), which also reported *Proteobacteria* as the most abundant phylum and suggested that their presence is a consequence of increased organic input by cave visitors. Considerable input of organic matter in many caves supported the growth of different types of heterotrophic bacteria, led to changes in microclimate and increased microbial dispersal and colonization (Ikner et al., 2007; Bastian et al., 2009; Mulec et al., 2012). Bat guano appears to be the major source of organic matter in Magura Cave that could contribute in increasing the abundance of *Proteobacteria* in the Gallery with the drawings. The predominance of *Serratia* and especially *Pseudomonas* isolates in Magura Cave could be considered as an evidence of a high degree of contamination of surfaces, probably due to insects and bats feces (Jurado et al., 2010), or could be the result of using rich medium for isolation. Comparing the bacterial diversity in caves from Australia, China, Spain and USA, Zhou et al. (2007) have found that a general trend in caves is that *Proteobacteria* is the largest group, followed by *Acidobacteria*, *Actinobacteria*, and *Firmicutes*. The bacteria isolated from several Siberian karstic cavities have been identified as *Pseudomonas*, *Arthrobacter*, *Bacillus*, and coryneform bacteria (Khizhnyak et al., 2003). Chelius & Moore (2004) have cultured *Actinobacteria* and *Proteobacteria* isolates from Wind Cave (USA), but have not recovered any *Firmicutes*. Bacterial communities in the cave Škocjanske Jame (Slovenia) contained *Enterobacteriaceae* and *Vibrionaceae*, low proportions of Gram-positive bacteria, and practically no culturable *Actinobacteria* (Gerič et al., 2004). Contrary to our findings for Magura Cave, *Actinobacteria* are a dominant group in heterotrophic bacterial communities in many caves (Groth & Saiz-Jimenez, 1999). Most of the isolated *Actinobacteria* could be assigned to the genus *Streptomyces* in certain caves such as Grotta dei Cervi (Italy), Altamira and Tito Bustillo (Spain), and 19 karstic caves in Turkey (Groth et al., 1999, 2001; Yucel & Yamac, 2010). The low number of isolated *Actinobacteria* from Magura Cave could be due to the use of nutrient agar/broth, which is not routinely used for their isolation. Three of the isolated strains from Magura Cave (IM6, IM10, and IM11) were representatives of the genus *Arthrobacter*. One isolate, IM13, belonging to the genus *Myroides* is a candidate for a new species (accession number HE663456) suggesting that Magura Cave could be a source of novel microorganisms.

Some of the species belonging to the genera, identified in Magura Cave: *Pseudomonas*, *Bacillus*, *Sphingobacterium*, *Stenotrophomonas*, *Arthrobacter*,

Table 2. Extracellular enzyme activities of heterotrophic bacteria isolated from the Gallery with the drawings in Magura Cave.

Isolate code	Strains referred to the nearest 16S sequence identity	PrA skim-milk	PrA gelatin	LipA Tween 80	AmA	RNAse	DNase	CMCase	Urease	PGase	XanthA*	PhytA	β-gluA
B1	<i>Enterobacter</i> sp. 2B1C	-	-	++	-	-	-	-	-	-	++	-	-
B2	<i>Pseudomonas fragi</i>	++	++	-	-	-	-	-	-	-	++	++	-
B3	<i>Pseudomonas fluorescens</i> 15834	+++	+++	+	-	-	-	-	-	-	+	+	±
B4	ND	+	-	-	-	-	-	-	-	-	+	-	-
B5	<i>Serratia</i> sp.	+	+	-	-	-	-	-	-	-	-	-	-
B6	<i>Enterobacter asburiae</i> JCM6051	±	-	-	-	-	-	-	++	-	-	-	-
B7	<i>Sphingobacterium</i> sp.	±	±	-	-	-	-	-	-	-	+	-	-
B8	<i>Pseudomonas</i> sp.	++	-	-	-	-	-	-	++	-	++	-	-
B9	<i>Bacillus safensis</i> KTT-25	+++	+++	-	-	-	-	-	-	-	+	-	±
B11	<i>Enterobacter</i> sp.	±	+	-	-	-	-	-	-	-	+	-	-
B13	<i>Sphingobacterium</i> sp.	++	±	-	-	-	-	-	+	-	+	-	-
B14	<i>Serratia</i> sp. L0305	+	+++	++	-	-	-	-	++	-	++	+	+
B16	<i>Serratia</i> sp.	+	++	+++	-	-	-	-	+++	-	-	-	-
B17	<i>Micrococcus luteus</i> C-J-G-TSA7	+	-	-	-	-	-	-	+++	-	++	-	++
B18	<i>Serratia</i> sp.	-	-	±	-	-	-	-	-	-	++	-	-
B19	ND	+++	+	±	-	-	-	-	-	-	+	-	-
B20	<i>Serratia</i> sp.	-	++	+	-	-	-	-	-	-	-	+	+
CB9	<i>Micrococcus luteus</i> CUB12	+++	+++	++	-	-	-	-	-	-	+++	+	-
CB11	<i>Serratia</i> sp.	-	++	±	-	-	-	-	-	-	+	-	+
CB12	<i>Bacillus flexus</i> SL21	+++	-	-	-	-	-	-	-	-	++	-	-
CB13	ND	++	-	+	-	-	-	-	-	-	++	-	-
CB15	<i>Sphingobacterium</i> sp. Ag8	+++	+++	++	-	-	-	-	-	-	-	+	+++
CB16	<i>Bacillus amyloliquefacien</i> ZP0308	+++	+++	-	++	++	-	-	-	-	-	-	-
G5	<i>Acinetobacter</i> sp. C-J-S-PYD4	-	-	++	-	-	-	-	-	-	+	±	±
G7	<i>Pseudomonas</i> sp.	++	+	-	-	-	-	-	++	-	-	+	-
IM1	<i>Serratia</i> sp. 136-2	+++	±	+	-	-	-	-	-	-	-	-	+
IM2	<i>Serratia</i> sp.	-	++	+	-	-	-	-	-	-	+	-	-
IM3	<i>Sphingobacterium</i> sp.	-	+++	-	-	-	-	-	-	-	+	-	-
IM4	<i>Serratia</i> sp.	+++	-	+	-	-	-	-	-	-	-	-	+
IM5	<i>Pseudomonas fluorescens</i>	+++	+	+	-	-	-	-	-	-	+	-	-
IM6	<i>Arthrobacter</i> sp. R4	++	-	-	-	-	-	-	-	-	++	-	-
IM7	<i>Obesumbacterium proteus</i> 511	-	±	-	-	-	-	-	-	-	+	-	+
IM8	<i>Serratia proteamaculans</i> DSM4543	-	-	+	-	-	-	-	-	-	+	-	±
IM9	ND	-	+++	-	-	-	-	-	-	-	±	-	+
IM10	<i>Arthrobacter</i> sp. R-36193	+++	+	+	-	-	-	-	-	-	-	-	++
IM11	<i>Arthrobacter</i> sp.	+	±	-	-	-	-	-	-	-	+	-	-
IM12	<i>Pseudomonas fluorescens</i> LMG 14576	+++	+++	-	-	-	-	-	-	-	±	-	±
IM13	<i>Myroides</i> sp. IT-2012	-	-	-	-	-	-	-	+	-	-	-	-
IM14	<i>Serratia</i> sp. a101-87	+++	++	-	-	-	-	-	-	++	-	±	++
IM15	<i>Pseudomonas putida</i>	+++	-	-	-	-	-	-	-	-	-	+	-
IM16	<i>Serratia</i> sp.	+++	-	+	-	-	-	-	-	++	±	-	++
IM17	<i>Stenotrophomonas</i> sp. DIC6JA	+++	+	-	-	-	-	-	-	-	-	-	+
IM18	<i>Comamonas</i> sp. BM-9_6	-	++	-	-	-	-	-	-	-	-	-	-
IM19	<i>Stenotrophomonas</i> sp.	+++	+++	-	-	-	-	-	-	-	-	-	+
IM20	<i>Pseudomonas plecoglossicida</i>	-	++	-	-	-	-	-	-	-	±	-	-
IM21	<i>Pseudomonas</i> sp.	-	-	-	-	-	-	-	-	-	+	-	-

Symbols: ±, zone < 1 mm; +, zone 1 to 3 mm; ++, zone 3 to 5 mm; + + +, zone > 5 mm. *Xanthan lyase activity was tested in liquid MSM with xanthan as a sole carbon source.

Table 3. Antimicrobial and hemolytic activity of heterotrophic bacteria isolated from the Gallery with the drawings in Magura Cave

Isolate code	Strains referred to the nearest 16S sequence identity	Inhibition zone (mm)				Hemolytic activity (mm)
		<i>B. subtilis</i>	<i>X. oryzae</i>	<i>P. aeruginosa</i>	<i>R. mucilaginosa</i>	
B1	<i>Enterobacter</i> sp. 2B1C	-	-	-	-	-
B2	<i>Pseudomonas fragi</i>	-	-	8	-	W
B3	<i>Pseudomonas fluorescens</i> 15834	-	2	20	16	W
B4	ND	-	-	8	-	W
B5	<i>Serratia</i> sp.	-	4	-	-	3.0
B6	<i>Enterobacter asburiae</i> JCM6051	-	-	-	-	-
B7	<i>Sphingobacterium</i> sp.	-	10	10	-	W
B8	<i>Pseudomonas</i> sp.	-	-	-	10	2.0
B9	<i>Bacillus safensis</i> KTT-25	-	-	-	-	-
B11	<i>Enterobacter</i> sp.	-	-	-	-	W
B13	<i>Sphingobacterium</i> sp.	-	-	12	6	-
B14	<i>Serratia</i> sp. L0305	-	-	10	6	W
B16	<i>Serratia</i> sp.	-	-	10	-	2.0
B17	<i>Micrococcus luteus</i> CJ-G-TSA7	-	-	-	2	W
B18	<i>Serratia</i> sp.	-	-	-	-	W
B19	ND	-	-	8	-	2.0
B20	<i>Serratia</i> sp.	-	-	-	-	W
CB9	<i>Micrococcus luteus</i> CUB12	-	-	-	-	-
CB11	<i>Serratia</i> sp.	-	-	-	-	W
CB12	<i>Bacillus flexus</i> SL21	-	-	-	-	W
CB13	ND	-	-	8	-	-
CB15	<i>Sphingobacterium</i> sp. Ag8	-	-	10	-	W
CB16	<i>Bacillus amyloliquefaciens</i> ZP0308	-	-	10	-	3.0
G5	<i>Acinetobacter</i> sp. CJ-S-PYD4	-	-	-	4	-
G7	<i>Pseudomonas</i> sp.	-	-	-	8	3.0
IM1	<i>Serratia</i> sp. 136-2	-	-	10	2	-
IM2	<i>Serratia</i> sp.	-	-	-	2	W
IM3	<i>Sphingobacterium</i> sp.	-	-	-	-	3.0
IM4	<i>Serratia</i> sp.	-	-	12	-	W
IM5	<i>Pseudomonas fluorescens</i>	-	-	12	16	3.0
IM6	<i>Arthrobacter</i> sp. R4	-	-	4	6	3.0
IM7	<i>Obesumbacterium proteus</i> 511	-	-	10	16	4.0
IM8	<i>Serratia proteamaculans</i> DSM4543	-	-	14	2	-
IM9	ND	-	-	14	4	W
IM10	<i>Arthrobacter</i> sp. R-36193	-	-	6	4	-
IM11	<i>Arthrobacter</i> sp.	-	-	-	-	2.0
IM12	<i>Pseudomonas fluorescens</i> LMG 14576	-	-	-	16	W
IM13	<i>Myroides</i> sp. IT-2012	-	-	-	8	W
IM14	<i>Serratia</i> sp. a101-87	-	-	-	8	3.0
IM15	<i>Pseudomonas putida</i>	16	6	-	8	W
IM16	<i>Serratia</i> sp.	16	2	12-14	10	2.0
IM17	<i>Stenotrophomonas</i> sp. DIC6JA	-	-	12-14	-	2.0
IM18	<i>Comamonas</i> sp. BM-9_6	20	10	12-14	8	3.5
IM19	<i>Stenotrophomonas</i> sp.	-	-	12-14	2	W
IM20	<i>Pseudomonas plecoglossicida</i>	20	10	4	10	3.5
IM21	<i>Pseudomonas</i> sp.	-	-	-	-	3.0

Symbols: -, no zone observed; clear inhibition zones minus 6 mm diameter of well are indicated. W, weak clear zone ≤ 1 mm

Acinetobacter, and *Enterobacteriaceae* family could be potential pathogens, perhaps translocated into the cave by animals and human visitors (Northup & Lavoie, 2001; Campbell et al., 2011). Bastian et al. (2009) reported that the Lascaux Cave is a reservoir of potential pathogenic bacteria. Pathogenic bacteria frequently isolated from caves are *Escherichia coli*, *Staphylococcus aureus*, and species of *Pseudomonas*, *Sphingomonas*, and *Alcaligenes* (Ikner et al., 2007). Lavoie & Northup (2006) considered *Bacillus* spp., *E. coli*, and *S. aureus* as indicators of human impact in the caves.

We found that most of the isolated heterotrophic bacteria from Magura Cave (about 69%) are psychrotolerant (psychrotrophic) by the highest growth rate observed between 20 and 30 °C. Similarly, Khizhnyak et al. (2003) reported that the amount of typically mesophilic strains in some Siberian cavities is many times lower than psychrophilic and psychrotolerant strains. According to Gounot (1994), all autochthonous bacteria in caves situated in temperate regions are psychrotrophic, growing well at 20 °C and above.

Psychrophilic and psychrotrophic microorganisms have developed a complex range of structural and functional adaptations to thrive successfully in low temperature environments (Margesin & Miteva, 2011). Poor levels of nutrients in the caves promote the competition of microorganisms by production of high amounts of exopolymeric substances, enzymes and antimicrobial metabolites that is one of the strategies for their survival (Smalås et al., 2000; Burgess et al., 1999; Slattery et al., 2001). It is known that heterotrophic bacteria and their enzymes play a key role in the processes of recycling, decomposition and mineralization of a wide spectrum of organic polymers in different environments (Munster & De Haan, 1998). Patterns of enzyme activities can be used to define the composition of organic matter in the ecosystems (Boschker & Cappenberg, 1998). Heterotrophic bacteria isolated from Magura Cave showed varied enzymatic patterns, which expressed different degrees of nutritional and ecological versatility.

Only few reports have been published on hydrolytic enzymes production by culturable bacteria isolated from subterranean environments including caves. Martinez et al. (2011) considered that measuring extracellular enzyme activity is one way to measure microbial responses to nutrient availability. The authors have assayed alanine aminopeptidase, phosphatase, and β -glucosidase activities in samples from three limestone caves in New Mexico and have determined whether microorganisms are nitrogen, carbon or phosphate limited. Similar to our results, Khizhnyak et al. (2003) reported that cultures from several Siberian caves synthesized more exogenous proteases compared to amylolytic enzymes because there were no natural sources of starch there. Rashid et al. (2001) reported for a low-temperature lipase produced by psychrotrophic *Pseudomonas* sp. isolated from subterranean environments. Psychrotolerant heterotrophic bacteria isolated from Magura Cave at given conditions could be attractive producers of

valuable cold-tolerant enzymes with a potential for application in various industries - in food and dairy industry, textile industry, brewing and wine industry, laundry, etc. High activity of cold-active enzymes at low and moderate temperatures offers potential economic benefits through substantial energy savings in large-scale processes (Russell, 1998; Gerday et al., 2000; Cavicchioli et al., 2011). Our study is a first investigation of production of RNase, urease, phytase, polygalacturonase, and xanthan lyase enzymes by psychrophilic/psychrotolerant microorganisms isolated from caves. As promising producers of cold-tolerant enzymes could be accepted: B2 (*Pseudomonas fragi*) and CB9 (*Micrococcus luteus*) – for xanthan lyase and phytase; B16 (*Serratia* sp.) – for lipase and urease; B17 (*Micrococcus luteus*) – for urease; CB15 (*Sphingobacterium* sp. Ag8) – for protease, phytase and β -glucosidase; CB16 (*B. amyloliquefaciens*) – for protease, amylase and RNase; *Serratia* strains IM14 and IM16 – for polygalacturonase and β -glucosidase.

Nowadays, there is a need for new antimicrobial agents due to the increase in drug resistance in many common bacterial pathogens, and the emergence of new infections (Davis & Webb, 1998). Cold-adapted microorganisms have been recognized as valuable sources of novel bioproducts including antimicrobial metabolites (Sanchez et al., 2009). Information about antimicrobial activity of bacteria isolated from karstic caves is rare, related mainly to *Actinobacteria* (Kim et al., 1998; Yucel & Yamac, 2010). Kim et al. (1998) have shown that up to 78% of *Actinobacteria* isolated from cave soils in Korea have been antagonistic to plant pathogenic fungi. About 62% of *Actinobacteria* isolated from rock wall and speleothems surfaces, and soil samples of 19 karstic caves in Turkey exhibited antimicrobial activity against a panel of four bacteria, two yeasts and four filamentous fungi (Yucel & Yamac, 2010). We selected several bacterial isolates from Magura Cave as promising producers of antimicrobial compounds such as B3 (*P. fluorescens*), B7, B13 (*Sphingobacterium* sp.), IM15, IM18 (*Comamonas* sp.), IM19 (*Stenotrophomonas* sp.), and IM20 (*P. plecoglossicida*). Isolates B5, B7, IM15, IM18, and IM20 exhibiting antibiotic activity against *X. oryzae* are prospective strains for future application in the agriculture for plant protection.

Most of heterotrophic bacteria isolated from Magura Cave exhibited hemolytic activity on blood agar plates. Surface activity is often accomplished by hemolysis and sometimes by antimicrobial activity, and both activity assays have been used to search for surfactant-producing bacteria. Due to their amphiphilic character, surfactants often induce hemolysis at a given concentration (Vinardell & Infante, 1999). Hemolysis, observed on blood agar plates, has been widely used to screen surfactant-producing microorganisms (Moran et al., 2002). Production of biosurfactants is of biotechnological importance due to their potential application in different industries - in food, cosmetics, pharmaceutical, agricultural, petrochemical industries, etc. (Banat et al., 2010). The search for different types of biosurfactants is important since their use in industry requires

different physicochemical properties. *Bacillus*, *Pseudomonas*, and *Serratia* strains are well-known producers of lipopeptide biosurfactants having important biotechnological applications, whereas knowledge about *Comamonas*, *Sphingobacterium* or *Stenotrophomonas* as producers of surface active substances is rare (Banat et al., 2010; Burgos-Diaz et al., 2011). Among microorganisms isolated from caves, only *Actinobacteria* have been screened for biosurfactant production (Bottos, 2004). Our study is a first report about the hemolytic activity of heterotrophic cave bacteria. Eight of bacterial strains isolated from Magura Cave (B5, CB16, IM5, IM6, IM7, IM16, IM18, and IM20) expressed high hemolytic activity together with antimicrobial activity and could be suggested as prospective producers of biosurfactants.

In conclusion, our study represents a first report on microbial diversity of a cave in Bulgaria. The results obtained showed that psychrotolerant aerobic culturable bacteria isolated from karstic Magura Cave could serve as a source of industrially important enzymes and bioactive secondary metabolites.

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