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ACTINOBACTERIA AS A COMPONENT OF MICROBIAL COMMUNITY FORMED ON ROCK PAINTINGS OF MAGURA CAVE, BULGARIA

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Abstract: The Magura Cave is located in Northwestern Bulgaria. The cave contains an impressive display of prehistoric paintings made of guano feces of cave-dwelling bats. Many different types of microorganisms may grow on such substrate under favorable environmental conditions and have a biodeterioration effect on the paintings. Among numerous biological agents, Actinobacteria play a critically important role in stone deterioration. They cause various types of damage to stone as a result of biofilm formation, physical penetration into the substrate and chemical reaction with the substrate by pigments.

The aim of the present study was to investigate the Actinobacteria diversity from samples derived from Gallery with paintings, Magura cave. For this purpose the isolates obtained were identified on the basis of morphological, physiological and biochemical characteristics. The molecular approach, including the amplification of 16S rDNA was also used. The antagonistic effect of the isolates as well as their sensitivity to different biocides was tested too.

The results showed that the isolates are closed to the genus *Streptomyces*. The isolates have relatively strong antagonistic effect against *Bacillus, Sphingobacterium, Sarcina* and *Pseudomonas,* which were used as test microorganisms. The biocides tested inhibited the isolates at concentration 1% and the most active biocide against these isolates is Cu^{2+}.

The results obtained are important for the development of correct conservation and restoration strategies of the unique rock paintings.

INTRODUCTION

The Magura Cave, Bulgaria is a karst cave situated in the North-western corner of Bulgaria, Vidin district. It's 25 km far from Belogradchik. Magura Cave is located a few km east of the Serbian border and 20 km west of the Danube, here marking the border with Romania.

The first description of the Magura Cave was made in 1887 by Dimitar Marinov. The archeologist Vasil Mikov first announced the cave drawings in 1927.

On the 3rd of May 1960 the Magura Cave was declared a natural landmark and was enlisted in the list of the preferred tourist sites. During the next two years it was developed and made accessible to the thousands of tourists.

In 1984 the site was placed on the Tentative List for consideration as a World Heritage Site by UNESCO. The popularity of the cave is mostly due to the "ancient paintings". On both sides of the Gallery of drawings and on the walls of the Sunny chamber there are around 700 drawings, divided into eight major groups. They depict schematic male and female figures, fantastic animals and signs, resemble letters from an unknown alphabet. They are made of bat guano.

Prehistoric cave paintings, some of them dating back 20,000 years, are a unique legacy of considerable importance. Because caves containing such paintings have drawn millions of visitors and attract considerable cultural and political attention, their preservation is a priority.

Prehistoric paintings are exposed to a variety of potentially damaging physical, chemical and biological factors. Among the latter, microorganisms are of special importance through colony and biofilm formation. In some cases, microorganisms may damage or destroy paintings by using them as substrates during growth, by producing destructive metabolites, or by covering them. Developing strategies for conserving prehistoric cave paintings depends on better understanding of the metabolic capabilities of the microbial communities colonizing those environments.

Actinomycetes were found in different caves during the last few years (Pašić et al., 2010; Northup et al., 2011; Cuezva et al., 2012; Niyomyong et al., 2012; Quintana et al., 2013; Barton et al., 2014; Hathaway et al., 2014). Moreover, many of these species were characterized (Groth et al., 1999; Lee et al., 2000, 2001; Jurado et al., 2005a,b; Lee, 2006). Cave actinomycetes are of a great interest because of the unique environment in which they survive. Extremal environment (e.g. low nutrients concentration, low primary production) force microbial communities to use different metabolic pathways (Cuezva et al., 2012; Miller et al., 2012a,b). Review of world literature show that little is known about the distribution, population dynamics, growth rates and biogeochemical processes of actinomycetes in the caves, despite the fact that they seem to represent a significant portion of the microbial population in these habitats.

Cave microbiology is strictly regulated by environmental factors. While most caves show fairly constant conditions throughout the year, changes in that equilibrium could result in fluctuations in the microbial community composition.

Strict management should be reinforced to maintain the natural conditions of this cave in order to prevent the negative microbial effects on its conservation.

The goal of this research is to characterize actinobacterial community on rock and sediment surfaces throughout the Gallery with paintings, Magura Cave and investigate the antagonistic activity of the isolates against bacteria. The sensitivity of the isolates to different biocides was also investigated.

MATERIALS AND METHODS

Sampling, Transportation, Preliminary manipulation

Sampling and samples preservation till further manipulation is done according to standard procedures for microbiological analysis (Bulgarian State Standards). Priority for sampling was given to locations with signs of possible biodeterioration activity (paintings itself, wet wall surfaces, spots with different color) (figure 1).

The samples were taken from different locations in the cavern area with sterile cotton swabs (Constix® Swabs, Contec, Canada).

Fig. 1. Sampling locations.

Isolation and identification of the actinomycetes

Swab samples were aseptically transported to the laboratory, where 2 ml of sterile 0,9% NaCl solution was added directly to them. After that, they were placed on shaker for 30 min. The samples were serially diluted by the method of serial dilution. Aliquots from each dilution were inoculated into plates with growth media. The cultivation was done at 25ºC ± 2ºC for 24-48 hours. Single colonies were used for isolation of the pure cultures. Identifications of the isolates obtained was based on classical microbiological scheme (Bergey'Manual of Systematic Bacteriology, 2nd Edition. George Carrity Ed.Springer-Verlang, New York, 2004; Bergey'Manual of Determinative Bacteriology, 9th Edition., John Holt Ed.Williams & Wilkins Ed., 2009) on the basis of morphological physiological and biochemical characteristics as well as International Streptomyces Project (ISP). The micro morphology of the isolates include few characteristics as shape and size of the spores, spore chain morphology and spore surface ornamentation. Light and scanning electron microscopy (SEM) were used. The macro morphological characteristics as colony morphology (including elevation, surface, density of the colony), aerial and substrate mycelium colour and pigment production was carried out too.

For molecular identification genomic DNA from strains was extracted according to the classical method for DNA extraction. Actinobacterial biomass was centrifuged at 4000 rpm for 15 min. (Centrifuge, type MPW-360 - Poland). 500µl T100 buffer and 50µl SDS (10%) were added to the cell biomass. The suspension was incubated at 65^0C for 15 minutes (Dry Block Thermostat Bio TDB-100 BIOSAN). After adding 300µl of 5M potassium acetate (KAC) the suspension was incubated at 40^0C for 20 min., followed by centrifugation at 13 000 rpm for 10 minutes at 4^0C . The supernatant was placed into a sterile Eppendorf quantitative test tube and the same amount of isopropanol (v/v) was added. After 10 minutes incubation at room temperature, the DNA was precipitated by centrifugation at 10,000 rpm for 10 minutes at 4^0C . The supernatant was removed, and the remnants of the isopropanol are removed by washing the DNA with 70% ethanol. The precipitated DNA was dried at room temperature to lightening, after that the pellet was resuspended in 50µl TE (x1) buffer. The isolated DNA was stored at 2 – 80^0C

The Actinobacterial 16S rDNA was amplified by PCR using primers (Strep B/ Strep E): Strep B, 5'-ACA AGC CCT GGAAAC GGG GT-3'/ Strep E, 5'-CAC CAG GAA TTC CGA TCT- 3' (Rintala et al., 2001; Suutari et al., 2001). PCR amplifications were performed in thermal cycler MJResearch/PTC-200. PCR reactions were performed in 50 µl volumes, containing: 5 µl 10x PCR buffer (STS Ltd.); 1.5 mM $MgCl_2$; 0.04 mM of each of dNTP; 0.4 U DNA Taq Polymerase (STS Ltd.); 10 pmol of each primers (STS Ltd.); 1µl of extracted DNA. PCR amplifications were performed using the following cycling parameters: 5 min of initial denaturing step at 94ºC, followed by 35 cycles of denaturing (94ºC for

45 s), annealing (56ºC for 45 s) and extension (72ºC for 45s), with an additional extension step at 72ºC for 7 min at the end. To evaluate the PCR results, all products were electrophoresed on 1,5% (w/v) agarose gels (VWR Prolabo) in 1x TBE buffer (30 min, 100 V), stained with ethidium bromide (AppliChem, GmbH) and visualized under UV light. DNA standard 100 bp was used (Fermentas). .

Antagonistic activity of the isolates

Determination of antimicrobial activities of the isolates was performed according to agar diffusion method (Perpendicular streak technique). Nutrient agar plates were prepared and inoculated with actinobacterial cultures by a single streak on the surfaces of the Petri dish and incubated at 28^{o}C for 7 days. The plates were seeded with test organisms by streaking perpendicular to the line of actinobacterial growth. The plates containing active organisms were kept for 14-28 days to determine whether the test bacterial strains possessed any further secondary growth towards the actinobacterial streaks. Four bacterial strains from genera *Bacillus, Sphingobacterium, Sarcina* and *Pseudomonas* isolated also from Gallery with paintings were used as test bacteria.

Tolerance of Actinobacteria to chemical agents

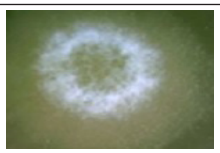
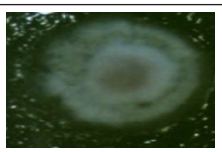
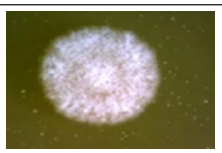
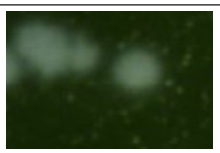
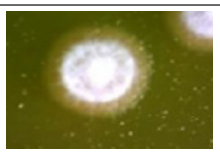
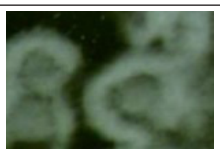
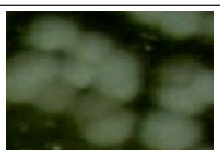
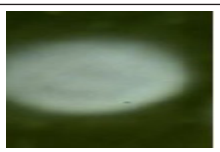
The agar diffusion assay method was used to quantify the ability of several chemical agents to inhibit actinobacterial growth. Three different biocides - thymol, silver nitrate and copper sulphate were tested. For each biocide three different concentrations - 0.1%, 0.5% and 1.0% were used.

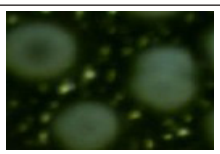
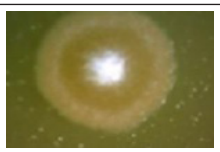
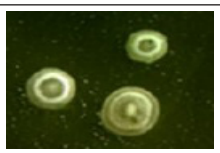
Petri dishes with cultivation media were inoculated with 200 µl actinobacterial suspension. 100 µl of each biocide with corresponding concentration was applied to a well that is cut into the agar. Analysis was performed in 6-9 replicates and one control with sterile water. The plates were incubated at 28^{0}C for 72 h. The zone of the inhibition of the growth around the wells was used as indicator of the antibacterial activity of each biocide tested..

RESULTS AND DISCUSSION

Fifteen pure cultures of actinobacteria from 6 samples taken of the rock paintings were isolated. These isolates have been subjected to a subsequent identification including morphological and biochemical characteristics. Different morphological types of colonies after growth on different media were observed (table 1).

Table 1. Macro morphological characteristics of the isolates.

Strains	Colony	Description
AM1		White, fluffy, aerial mycelium. Well defined flat center, covered with a loose aerial mycelium. White substrate mycelium does not release pigment in media.
AM2		White, fluffy aerial mycelium. Whitish substrate mycelium. Flat center with white fluffy aerial mycelium around it. Release whitish pigment in media.
AM3		Whitish substrate mycelium; release pigment in media; conical colonies with light pink aureole at the base; dark pink- gray tops and white fluffy aerial mycelium.
AM4		White aerial mycelium. Brown- tiled substrate mycelium. convex white center with a whitish circle around it. Released brown tile pigment in media.
AM5		Substrate mycelium - indeterminate color, white and fluffy colonies, white aerial mycelium.
AM6		White fluffy aerial mycelium and dark brown substrate mycelium. White rough edge. Convex center with air mycelium. Surrounded by brown circle. Release brown pigment in media
AM7		White, fluffy aerial mycelium. Whitish substrate mycelium. Flat center with white fluffy aerial mycelium around it. Release whitish pigment in media
AM8		Released pigment, whitish to white substrate mycelium, aerial mycelium white, small white round colonies with a bright aureole side on top with darker part.
AM9		Released pigment; substrate mycelium – without distinguishable colour;

AM10		Released pigment, substrate mycelium without distinguishable color. Aerial mycelium - white colonies with a recess in the middle.
AM11		Orange substrate mycelium, release pigment in media, regular conical colonies with white fluffy aerial mycelium
AM12		Released brick-brown pigment in media. Brown substrate mycelium. White fluffy aerial mycelium. Convex center with white aerial mycelium on it. A light brown edge.
AM13		Convex colonies with greyish aerial mycelium, white on the edge and in the middle dark gray. It does not release pigment into media
AM14		White, fluffy, aerial mycelium, whitish substrate mycelium, convex colonies, toothed
AM15		Hairy aerial mycelium. Convex center with white top in the middle. Release yellow pigment in media

The colour of aerial mycelium was studied. Analysis of the results shows a significant variation in the colour of aerial mycelium in the tested isolates, depending on the used media. Grey colonies were dominant. There is also coloration in the darker range - grey-violet and dark violet (strains AM6, AM8, AM14) and nuances of yellow - AM11.

Variations in the colour of the substrate mycelium were also observed.

The data on the physiological, biochemical and morphological characteristics are subjected to the cluster analysis to group the strains on the basis of their similarity. The isolates were grouped into two clusters as follow:

1. Strains AM1, AM2, AM3, AM4, AM5, AM6, AM7, AM8, AM9 and AM10 formed the biggest group. The sporangiophores were mainly curved or open spirals. Most of them release soluble pigment in media. Aerial mycelium is predominantly white, gray-white. Substrate mycelium is mostly coloured in shades of brown. These isolates utilized the largest number of carbon sources.

2. Strains AM11, AM12, AM13, AM14 and AM15 formed separated group. Common for this group is ability to produce soluble brown-orange pigment, inability to produce melanin pigment and inability to utilize large number of carbon source.

On the basis of the results it was supposed that nine of the isolates probably belong to the genus *Streptomyces*, four isolates to genus *Kocuria* and two of them to genus *Promicromonospora.*

16s rDNA PCR with Streptomyces primers

For confirmation of the status of the isolates to genus *Streptomyces* 16S rDNA amplification with primers for *Streptomyces* was performed. In 10 of 15 isolated and analysed actinobacterial strains, an amplified product with 520 bp length, typical for genus *Streptomyces* (figure 2) was acquired.

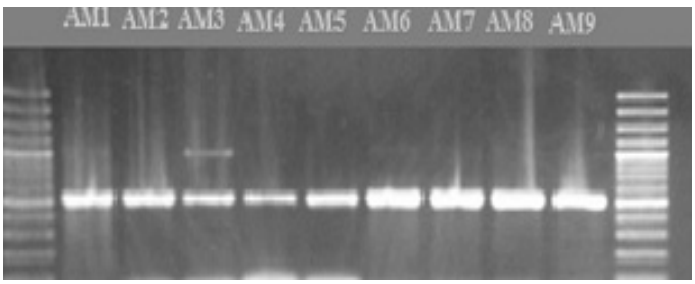


Fig. 2. 16s rDNA PCR amplification with *Streptomyces* primers (StrepB и StrepE).

Additional investigations as well as sequencing and bioinformatics analysis must be done for exact determination of the taxonomic status of the isolates. In any case actinobacteria from genera mentioned are found as component of microbial community in other caves and can play dominant role in microbial community formed (Barton et al., 2014; Hathaway et al., 2014).

Antagonistic activity

Antagonistic effects of the isolates against bacteria, isolated also from the gallery of paintings were tested. Eleven of the isolates did not show any antagonistic effect against the tests used. Strain AM2 showed effect against test microorganisms from genera *Bacillus, Sphingobacterium*, *Sarcina* and

Pseudomonas. Strain AM4 inhibited growth of the all test used but is most pronounced against *Bacillus.* Strain AM7 inhibited test microorganisms from genera Bacillus, *Sphingobacterium* and *Sarcina.* Antagonistic effect against test microorganisms from genus *Pseudomonas was not* observed. Strain AM9 possessed strongly expressed antagonistic effect against test microorganisms of the genus *Bacillus*, less expressed effect against genus *Sphingobacterium* and *Sarcina* and no inhibition of the tests from genus *Pseudomonas* (figure 3).

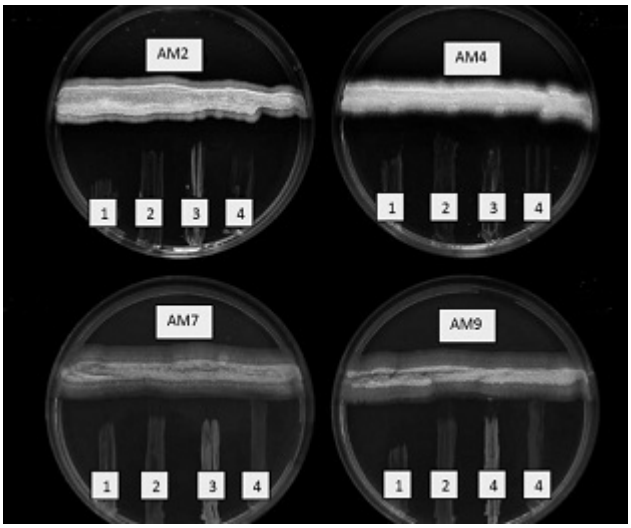


Fig. 3. Antagonistic activity of the isolates against bacteria, isolated from the rock paintings.
Legend: 1 – *Bacillus*; 2 – *Sphingobacterium*; 3- *Sarcina*; 4- *Pseudomonas*

Biocides tolerance

In order to establish the most effective substance for the treatment of actinomycetes, colonizing the paintings in Galllery, all isolated actinomycetes were treated with three different bactericidal compounds - thymol, silver nitrate and copper sulfate in different concentrations 0.1%, 0.5% and 1.0%).

Analysis of the results shows that the isolated actinomycetes are not sensitive to the biocides used in a concentration of 0.1%. Concentration of 0.5% inhibited development of the isolates, but the inhibitory effect is not significant. Apparent growth inhibition was observed at 1% concentration (figure 4).

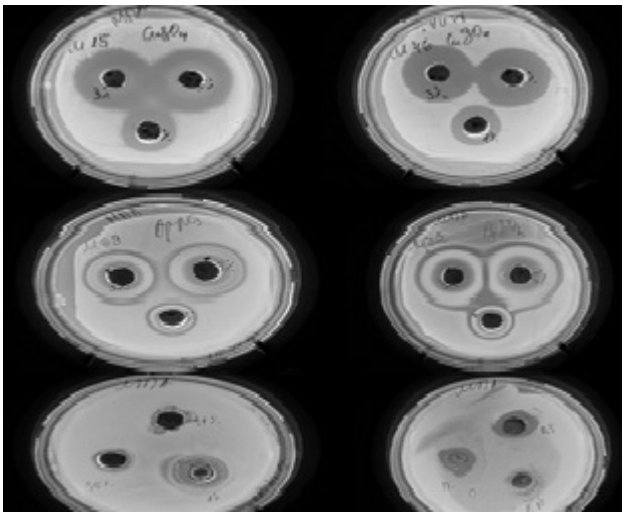


Fig. 4. Inhibition of actinobacterial growth by using biocides..

The most significant bactericidal effect was shown for Cu^{2+}. Silver nitrate at the same concentration has also expressed bactericidal effect. The thymol was not so effective against the isolates tested.

CONCLUSIONS

The analysis of the results consigns several conclusions. Actinobacteria are permanent component of the microbial community formed in the painting Gallery of Magoura Cave. The isolates differed significantly in their micromorphology and macromorphological characteristics as well as in utilization of different carbon sources. Arabinose and xylose are the most preferable carbon source for all the isolates. Most of the strains studied belong to genus *Streptomyces* which was confirmed by *16s rDNA PCR.* The genera *Kocuria* and Promicromonospora are also present as component of the microbial community.

Few of the isolates have relatively strong antagonistic effect against strains of the genera *Bacillus, Sphingobacterium, Sarcina* and *Pseudomonas,* which were used as test microorganisms. The biocides show low antibacterial activity against isolates tested at 0.1 % concentration. Growth inhibition was observed at 1% concentration of thymol, silver and copper ions. The isolates possessed sensitivity to silver and thymol, but the highest bactericidal effect was shown by copper ions (1%).

The results obtained are important for effective conservation of the unique rock paintings.

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