Identification of *Bacillus* strains isolated from rock paintings in Magoura cave, Bulgaria

**ABSTRACT**

Magoura Cave (Bulgaria) is one of the caves with Paleolithic paintings. An assessment of the composition of bacterial communities that have colonized this cave represents a first step in understanding and potentially controlling their proliferation in order to protect the paintings. The members of the *Bacillus* genus are generally found in caves and represent a wide range of physiological abilities, allowing them to grow in every environment. These bacteria compete desirably with other microorganisms within the environment due to its capability to form extremely resistant spores and produce metabolites that have antagonistic effects on other microorganisms. Many *Bacillus* species are of remarkable importance because of the potential of *Bacillus* species to synthesize a wide variety of metabolites which can take part in deterioration of rock paintings. The aim of this study was connected with the identification of *Bacillus* strains isolated from different samples of Paintings gallery. The identification of the isolates was achieved by the methods of classical and molecular taxonomy. The results showed that the strains are belonged to the species of *B. lutimorbus*, *B. licheniformus*, *B. macerens*, *B. validus*, *B. lautus*, *B. amyloliticus*, *B. polymyxa*, *B. insolutus* and *B. circulans*. A better understanding of the diversity of the genus *Bacillus* present on art objects is important for the development of effective conservation and restoration strategies of the unique rock paintings.

**Keywords:** *Bacillus*, rock paintings, deterioration, conservation

**Introduction**

The Magoura Cave is located in northeastern Bulgaria, 17 km from Belogradchik, in the limestone Rabisha burial mound (altitude 461 meters). The Magoura Cave was recognized as a natural landmark by Decree 666 on 3 May 1960. It is one of the largest and most beautiful caves in Bulgaria. It consists of the main gallery and three side branches. The overall length of the cave is approximately 2500 meters. The temperature in the cave is almost constant (around 12°C).

In one of the caverns prehistoric paintings have been discovered, carved into the walls and decorated with bat guano (droppings). The paintings depict the silhouettes of women, men dancing and hunting, animals, stars, tools and plants. The paintings date from different eras – the early Paleolithic, the Neolithic, the later Neolithic and the beginning of the Bronze Age. A solar calendar from the late Neolithic found there is the earliest solar calendar discovered in Europe. All over rock paintings are the most valuable part of our ancient cultural heritage, as it has witnessed the presence of prehistoric civilization, their respective sense of creativity and artistic abilities. The rock painting tradition is very old, dating back to prehistoric times and is also considered as one of the fine arts, practiced by early man to decorate their residential vicinity, usually cave shelters. The rock paintings also help us to understand about the contemporary societies, which reflect the pride of that particular nation's cultural heritage by its overall inventory. For this reason Magoura 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 the so called Gallery with the drawings was closed for visitors as the prevention of valuable drawings. The importance of proper protection of such rock paints hardly needs explanation. Numerous prehistoric caves have been naturally closed for thousands years having a stable microclimate, disconnected from the outdoor environment and casually discovered in the last two centuries. This closure and the isolation from the outer environment is one of the main reasons for the conservation of rock paintings. However, the opening to the public has dramatic consequences on their microclimate (Gonzalez et al., 1999). Caves are usually oligotrophic environments where primary production depends on autotrophic bacteria (both chemo- and photoautotrophs) (Cañaveras et al., 2001). However, due to the favorable microclimatic conditions, well established autotrophic communities can support the growth of several chemoorganotrophic microbes also thanks to inputs of organic matter from above ground (Urzì et al., 2003). Moreover, the distribution and the ratio of photoautotrophic and heterotrophic bacteria are influenced by the availability of nutrients or organic material (Chelius et al., 2009). In the last 20 years, several papers have been published on microorganisms colonizing caves of cultural value (Gonzalez et al., 1999; Groth et al., 2001; Jurado et al., 2007), and new chemoorganotrophic bacterial species have been isolated and described (Groth et al., 2006; Jurado et al., 2006; Urzì et al., 2008) showing a microbial diversity only partially known.

With the changes of environmental conditions in the caves, autochthonic microflora was replaced by other genera, mainly Bacillus, Micrococcus, etc. (Gonzalez et al., 1999), which are the bacteria commonly found in soils and soil airborne particles. It was assumed that the distinct environmental conditions in the caves produced different microbial communities and biogeochemical processes which makes a study interesting, and those bacteria colonizing the rock surfaces could be similar to those found in airborne soil particles. It is well known that mural paintings are colonized by microorganisms and can be severely damaged by such growths (Ciferri, 1999). In the cultivation studies on deteriorated mural and rock paintings, bacterial isolates have frequently been attributed to the genus Bacillus (Gonzalez et al., 1999; Saiz-Jimenez & Laiz, 2000). These bacteria compete desirably with other microorganisms within the environment due to its capability to form extremely resistant spores and produce metabolites that have antagonistic effects on other microorganisms. Many Bacillus species are of remarkable importance because of the potential of Bacillus species to synthesize a wide variety of metabolites which can take part in deterioration of rock paintings.

In this paper, the some data for taxonomic status of ten bacterial strains isolated from different parts of Paintings gallery, Magoura cave are presented.

**Materials and Methods**

**Sampling**

The bacterial strains have been isolated from Gallery with paintings. Sampling points are shown in the Figure 1. The strains are isolated from water, sediments, guano and paintings as it shown in the Table 1.

**Figure 1. Sampling points in the gallery with paintings.**

**Culture media and culture isolation**

A few media for isolation and characterization of the isolates as nutrient agar, nutrient liquid broth, phenyl ethyl alcohol agar (PEA), motility test medium (MM), minimal medium for Bacillus (MMB) were used. The plates were incubated at 28°C, in aerobic conditions. The strains were maintained at selective media with relevant energy source and were stored at 4°C.

**Characterization of the isolates**

The isolates were characterized by morphological, physiological and biochemical properties according classical taxonomy. Six morphological, 10 physiological and 18 biochemical characteristics were tested.
**Cluster analysis**

Cluster analysis was performed with dendrogram programmes from MIDI.

**DNA extraction and PCR**

DNA was extracted using the Prep Mini Spin Kit (GE Healthcare) according to the manufacturer’s instructions. 16S rDNA amplification was carried out using primer pair BK1/F (Sense) and B-K1/R1 (Antisense) (Xi-Yang et al., 2006).

**Table 1. Type of the samples for isolated strains.**

<table>
<thead>
<tr>
<th>Isolate</th>
<th>Type</th>
<th>Location</th>
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<tbody>
<tr>
<td>SB1</td>
<td>Water sample</td>
<td>The Paintings Gallery</td>
</tr>
<tr>
<td>B2-1</td>
<td>Sediment samples/guano</td>
<td>The Paintings Gallery</td>
</tr>
<tr>
<td>B2-2</td>
<td>Sediment samples/guano</td>
<td>The Paintings Gallery</td>
</tr>
<tr>
<td>B3</td>
<td>Sediment samples/guano</td>
<td>The Paintings Gallery</td>
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<td>B4</td>
<td>Paintings the Paintings Gallery</td>
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<td>B5</td>
<td>Paintings the Paintings Gallery</td>
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<td>B6</td>
<td>Paintings the Paintings Gallery</td>
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<tr>
<td>B7</td>
<td>Sediment samples/guano</td>
<td>The Paintings Gallery</td>
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<tr>
<td>B8</td>
<td>Paintings the Paintings Gallery</td>
<td></td>
</tr>
<tr>
<td>B9</td>
<td>Sediment samples/guano</td>
<td>The Paintings Gallery</td>
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</tbody>
</table>

**Results and Discussion**

For studying the morphology of the strains different staining were done. The results of the microscopic analysis showed that all isolates are typical rods with rounded ends and size between 1-3 μm. The isolates are typical spore-forming bacteria. The position of the endospores formed differed. Seven of the isolates possessed centrally placed endospores and in three of the strains the endospores are terminal. The strains are typical Firmacutes (Figure 2). Isolates are tested for growth on Nutrient Liquid Broth, PEA and MM. In liquid media the growth is abundant, mostly on the surface. During the growth of three isolates (B3, B5, B8) is observed discoloration of the media and slight change in the color. Isolate SB1 form precipitates. Analysis of the results of the oxygen demands, provides reasons to be consider that the isolates are typical aerobic microorganisms and facultative anaerobic. For analyzing the growth at different temperature isolates were inoculated at Nutrient Broth and PEA, and were incubated at different temperature - 10, 15, 20, 28, 37°C. The results obtained showethat all are mesophilic or facultative psychrophiles because growth well at 10 and 28°C. The strains are not halophilic and are typical chemoorganotrophs.

**Figure 2. Light microscopy of the isolates.**

The analysis of the results from the phenotypic characteristics tested showed that the isolates are members of the genus *Bacillus*.

According the cluster analysis done the isolates are highly different from those used as reference species. The dendrogram of the cluster analysis group isolates into three independently clusters with low similarity with the used references. Isolates B7, B4, B9 are close to *B. lentimorbus*, *B. licheniformis* and *B. macerans*. Isolates SB1, B3, B6 are close to *B. validus*, *B. lautus*, *B. amyloliticus*. These species are part of *Bacillus circulans* senso group. Isolates B2_2, B8, B5 are close to *B. polymixa*, *B. insolatus* and isolate B2_1 is close to *B. circulans* (Figure 3).

A better understanding of the diversity of the genus *Bacillus* present on art objects is important for the development of effective conservation and restoration strategies of the unique rock paintings. After standard PCR of the DNA isolated from the strains it was confirmed that are typical members of the genus *Bacillus* and selected primer sequences allow reliable identification of the natural isolated to the genus *Bacillus* (Figure 4).

According others authors application of different methods of the molecular taxonomy must be used in the case for identification of microbial diversity into the caves (Gurtner et al., 2000). Such investigations are going at the present because a better understanding of the diversity of the genus *Bacillus* present on art objects is important for the development of effective conservation and restoration strategies of the unique rock paintings in Magoura.
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Figure 3. Dendrogram from cluster analysis of the strains tested.

Figure 4. Electrophoretic profile of products from generic-specific amplification of isolates tested (1010 bp).

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References


