MICROBIAL COMMUNITIES IN CAVES: ECOLOGY, PHYSIOLOGY, AND EFFECTS ON PALEOLITHIC PAINTINGS

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Abstract

In this review we report on the culturable and non culturable microbial communities present in caves. A high diversity was found using both methods, however, identification methods including PCR amplification of 16S rRNA genes (16S rDNA) and community fingerprinting by denaturing gradient gel electrophoresis (DGGE) resulted into a greater bacterial taxonomic diversity, and allowed the detection of unexpected and unknown bacteria. Growth of cave bacteria at different temperatures was investigated. The data indicate that most cave bacteria can grow at temperatures in the range 5-45°C. However a higher diversity was obtained when incubation was carried out at 13°C, although incubation at this temperature drastically reduced the number of sporoactinomycete isolates. At 13°C the bacteria oxidized on average 87% of carbon sources provided while incubation at 28°C reduced to about 50% carbon source utilization. Selected actinobacteria isolates were tested for the formation of crystals. Strains from all tested genera except isolates of Gordonia and Nocardia produced vaterite and/or calcite. Production of Mg-calcite was restricted to strains of Brachybacterium, Paenibacillus, Rhodococcus and Streptomyces, while struvite was only precipitated by an unidentified isolate. These findings indicate that actinomycetes may play a role in the formation of mineral deposits in caves.

Keywords: Cave bacteria, actinobacteria, substrate utilisation, crystal formation
Introduction

In nature, chemical dissolution caused by naturally acidic waters transformed layers of permeable limestone into a dense network of tunnels, labyrinths and large halls, most of which are adorned by stalactites and stalagmites, which form the karstic system. These subterranean karstic systems of horizontal caves and vertical abysses are distributed all around the world. Only a limited number of these caves are actually accessible as show caves, from which a few contain Paleolithic and Neolithic parietal art.

The Mediterranean Basin is characterized by the presence of a huge number of karstic caves. There are over 7000 karstic cave formations in Greece, the majority in Crete. To date over 6500 such caves have been explored in Slovenia and there are probably as many which are, as yet, unexplored. Many Mediterranean Basin countries have caves with Paleolithic paintings, such as the well-known Altamira, Tito Bustillo, Lascaux, Chauvet, Niaux, etc., located in Spain and France. Some others like Grotta dei Cervi, Italy, contains valuable Neolithic paintings.

In spite of some available studies on geological, geochemical and hydrological characteristics (Hoyos et al. 1998; Sanchez-Moral et al. 1999), there are very few studies on cave microbiology, most of them in connection with the conservation of paintings (Hoyos et al. 1998; Groth et al. 2001).

Certainly microorganisms play a role in cave ecosystems, as they are able to colonize bare rock surfaces, use the low organic matter present in dripping waters and interfere in mineral crystallization processes. Cañaveras et al. (1999) suggested that bacteria present in caves may play a role in the formation of moonmilk deposits, as microbial communities predominantly
composed of different species of the genus *Streptomyces* were found in association with hydromagnesite and needle-fiber aragonite deposits in the Altamira Cave.

Caves are not uniform environments in terms of geological and geochemical characteristics, as they can vary from one to another. Show cave managements produce striking differences as well. Altamira and Lascaux caves have been throughout visited in the past, but had to be closed due to the deterioration observed in the paintings. Nowadays, visitors are derived to cave reproductions. Cave management tends to reduce these anthropogenic impacts by controlling visitors and microclimate (Hoyos et al. 1998).

In the last years several show caves were investigated in detail. These include Altamira, Tito Bustillo, La Garma and Llonin caves in northern Spain and Grotta dei Cervi in Porto Badisco, Italy (Groth and Saiz-Jimenez, 1999; Groth et al. 1999, 2001; Laiz et al. 1999, 2000). In this review we report on the ecology and physiology of cave bacteria.

**Cave biodiversity**

Actinobacteria have been considered to be mainly confined to soils (Porter 1971). However, it has been reported that they are found in nearly all natural habitats inclusively hypogean environments (Monte and Ferrari 1993; Groth and Saiz-Jimenez 1999).

Phototrophic microorganisms (cyanobacteria and algae) were absent in most studied caves due to the lack of light, actinobacteria growth was distributed all over the cave and could be observed on the active stalactites, on
upper and lower parts of the rock walls and in the cave soils. This seems to be a natural colonization, although, apparently, at least in some cases, actinobacteria could be related to anthropogenic disturbances, as colonies were observed at the surface of the archaeologist’s soil profile in Grotta dei Cervi.

In caves, samples of active stalactites, wall concretions and rocks from the walls and ceiling of the galleries were investigated and a high number of isolates was obtained. Thirteen seven genera were found among cave isolates (Table 1). The most abundant genera were assigned to the Actinobacteria division, particularly in rock walls and soils (Figure 1). In this division, *Streptomyces* spp. represented a large part of the isolated species (Figure 2). However, in stalactites, the most abundant species belonged to the low G+C Gram-positive bacteria of the genus *Bacillus*, although the most conspicuous and visible to naked eye were actinobacteria.

Actinobacteria are well known for their ability to grow on very poor media but also for their ability to use recalcitrant organic matter, e.g. lignocellulose residues, humic substances, etc. (Groth and Saiz-Jimenez 1999). While guano input is appreciable in the ceiling and walls of the galleries and in the cave soils of Grotta dei Cervi, this is not the case in other caves where organic matter was mainly present in dripping waters (Saiz-Jimenez and Hermosin, 1999).

The poor content of organic matter was noticed in the dripping water from stalactites, which increased during the spring season. However, considerable colonisation was observed on the Grotta dei Cervi stalactites (Laiz et al. 2000). The pattern of the actinobacteria colonies originating from stalactites was similar to cooperative growth patterns reported for other bacteria, and described by Ben-Jacob et al. (1992). This colony morphology is often combined with
unfavourable environmental conditions and the bacteria have to develop sophisticated modes of cooperative behaviour which seems to be an adaptative response to poor flow of nutrients. As it is shown in Figure 3 this type of growth was successfully reproduced in the laboratory with selected isolates. Groth and Saiz-Jimenez (1999) stressed that microbial communities of some caves usually rely on allochthonous input of dissolved and particulate organic matter which is transported from the surface.

It has been reported that the actinobacteria isolated from the Altamira cave revealed a great taxonomic diversity with the predominant isolates belonging to the genus *Streptomyces*. Members of the genera *Nocardia, Rhodococcus, Nocardiodes, Amycolatopsis, Saccharothrix, Brevibacterium, Microbacterium* and coccoid actinobacteria (family *Micrococcaceae*) were also found (Groth et al. 1999). The Grotta dei Cervi has a similar microbiological colonization but the biological diversity was somewhat higher. In spite of the facts that this cave was discovered in 1970, 91 years later than Altamira and that the total number of visitors since its discovery was very low (only for study purposes), its microbial colonization was surprisingly high. This probably could be due to the high content and the nature of the organic matter present in the cave (guano). Similar level of colonization was also noticed in La Garma cave, discovered in 1995 and visited in 1998. In this period only 64 persons with a total of 45 hours of stay in the cave were recorded. Therefore, bacterial colonisation seems to be independent from visits.

The studies on cultivated microorganisms in caves most probably reveal just a very minor and not representative part of cave population. No information is available on uncultivated microorganisms in these caves, except some
recently published papers (Schabereiter-Gurtner et al. 2002 a,b). For analysis, samples were directly taken from the Paleolithic painting area in Altamira and Tito Bustillo. Without prior cultivation, nucleic acids were extracted from the sampled material and analyzed the phylogenetic relationship of PCR amplified and cloned 16S rRNA genes (16S rDNA). Genetic fingerprinting of the bacterial community was performed by denaturing gradient gel electrophoresis (DGGE), which allows the sequence-specific separation of partial 16S rDNA amplicons of same length in polyacrylamide gels containing a denaturing gradient. 16S rDNA clone libraries were screened by DGGE and revealed phylogenetic information on individual bacterial members. The applied approach gave insight into a great bacterial taxonomic diversity, and allowed the detection of unexpected and unknown bacteria. Bacteria identified in Altamira and Tito Bustillo caves were related to members of the cosmopolitan Proteobacteria, to members of the Acidobacterium division, Cytophaga/Flexibacter/Bacteroides phylum, green non-sulfur bacteria, Planctomycetales and Actinobacteria. The high number of clones most closely related to environmental 16S rDNA clones showed the broad spectrum of unknown and yet to be cultivated bacteria in these caves (Figure 4).

Effect of temperature on bacterial growth

The almost constant low temperature throughout the year in most of the studied caves suggested the possibility of an indigenous psychrotrophic microflora adapted to low temperatures which could be overlooked using standard microbiological procedures and incubation at higher temperatures (28°C). To investigate this possibility, soil samples from three different northern
Spain caves (Tito Bustillo, La Garma and Llonin) were collected and studied at four different temperatures: 5, 13, 20 and 28°C. Enumeration, characterization of isolated species and substrate utilization pattern analysis were carried out to investigate the influence of the different incubation temperatures on the isolates and their physiological versatility.

Incubation at the four different temperature showed clear differences in viable cell counts (cfu). Cell counts at 28°C generally yielded two- to fourfold higher counts of cfu than at 5° or 13°C while count differences between 20° and 28°C were comparatively lower (Figure 5).

A total of 101 strains were isolated, 9 from Llonín, 21 from La Garma and 71 from Tito Bustillo. Only 29 isolates corresponded to sporoactinomycetes, most of them isolated from Tito Bustillo at different temperatures. At 13°C only 6 sporoactinomycetes were isolated from Tito Bustillo, but at 28°C the figure was tripled, indicating that isolation of actinobacteria is temperature-dependent. This agreed with literature data indicating that temperature optimum is 25-35°C, although some species grow at temperatures within the psychrophilic and thermophilic range (Holt et al. 1994; Groth et al. 1999). The relevance of actinobacteria in this cave was previously reported (Groth et al. 1999) and this high number of isolates is likely related to the input of organic matter and clays through periodic subterranean river floodings.

The most representative isolates obtained at different temperatures were selected for a study on the optimal growth temperature. Temperatures ranged from 5 to 45°C. The bacteria included two isolates from 5°C (*Kocuria* sp. and *Pseudomonas* sp.), four from 13°C (*A. viscosus, Bacillus* sp., *P. putida* and *S. ureae*), one from 20°C (*Bacillus megaterium*) and one from 28°C (*B.
halodurans). Sporoactinomycetes were not included in the study due to their clear response to temperature isolation. The isolates showed different optimal growth temperatures. At 24 h B. halodurans and Bacillus sp. had optimal growth at 28°C, while optimal growth for the rest was obtained at 38°C. At 5°C B. halodurans, B. megaterium and S. ureae failed to grow while the growth of A. viscosus, Bacillus sp., and P. putida was very poor. At 45°C all isolates grew, some with good growth (A. viscosus, B. megaterium and Kocuria sp.) Independent of the optimal growth temperature, the data show that these bacteria are able to grow comparatively well in a range of temperatures from 13º to 45°C.

Changes in fatty acid profile reflect an adaptative response to temperature. Variations with temperature were more pronounced for the fatty acid 16:1 w11c in Gram-positive and for the fatty acid 18:1 w7c in Gram-negative bacteria. Figure 6 shows the production of these fatty acids for selected isolates at different temperatures. Gram-positive bacteria increased up to 16 times the production of 16:1 w11c at 5°C when compared with growth at 38°C (e.g. A. viscosus) while S. ureae increased 12 times the production when compared at 13 and 38°C. For Gram-positive bacteria the increase in unsaturated fatty acid production appeared at 13°C while for Gram-negative bacteria this increase (less apparent than for Gram-positive bacteria) started at 20°C.

Substrate utilisation

A substrate diversity study was carried out with representative isolates covering three temperatures (Figure 7). The isolates were inoculated onto three
Biolog GN microplates and each microplate was incubated at 5, 13 and 28°C, respectively.

During incubation at their isolation temperature, the isolates from 5°C (Kocuria sp. and Pseudomonas sp.) oxidized numerous carbon sources. The higher number of carbon sources oxidized by these isolates occurred at 13°C. Kocuria sp. oxidized at 28°C an intermediate number of carbon sources while Pseudomonas sp. oxidized similar number of carbon sources either at 13 or 28°C. The rest of isolates were not tested at this temperature as no or poor growth was obtained in most of them.

The isolates from 13°C were the most active when incubated at their isolation temperature. In fact, Bacillus sp., S. ureae, A. viscosus, and P. putida metabolized, respectively, 91, 90, 89 and 62 out of 95 carbon sources in comparison with 33, 3, 6 and 49 at 28°C.

The isolate at 20°C (B. megaterium) demonstrated a higher carbon source utilization at 13°C than at 28°C, while the isolate from 28°C (B. halodurans) was the only case where a more efficient carbon source utilization at 28°C occurred.

It is remarkable that at 13°C a high number of carbon sources was utilized. In fact, on average, the tested bacteria oxidized 92% of all the carbohydrates, 82% of all the carboxylic acids, 88% of all the amino acids and 84% of all other carbon sources. In contrast to these results, carbon sources utilized at 28°C showed lower efficiency (57, 42, 47 and 58%, respectively).

From the study of Biolog GN microplates, it appears that most bacteria isolated in the range 5-28°C oxidize carbon sources at incubation temperatures between 13 and 28°C, showing a wide metabolic versatility. The temperature of
most northern Spain and southern France caves is 13°C, the temperature at which a higher number of carbon sources is oxidized, thus indicating a high physiological adaptation. This suggests that cave bacteria can be very active in situ.

The oxidation of a higher number of carbon sources at 13°C could be explained by the fact that cave bacteria have the ability to alter membrane lipid composition for an efficient nutrient utilization at low temperatures (Russell, 1990, 1993). This can be observed in whole-cell fatty acid production at different temperatures, with an increase of unsaturated fatty acids in the lowest temperature range. Increasing of unsaturation of fatty acids is the most frequently reported response of Gram-negative bacteria to decreasing temperatures (Männistö and Puhakka, 2001).

Growth at low temperature seems to be correlated with a modulation of the outer membrane permeability. Dé et al. (1997) indicated that the response of *Pseudomonas fluorescens* to a temperature that falls under its optimum growth temperature is a decrease of the outer membrane permeability to avoid the entry of toxic molecules. This effect is counterbalanced by the overproduction, at low temperature, of exocellular enzymes (lipases, proteases, etc.) for degrading macromolecules into small units which provides *P. fluorescens* with carbon and energy sources.

**Crystal formation**

A variety of different hygroscopic salts, including carbonates, chlorides, nitrates, sulphates can be found in caves (Cañastras et al. 1999). Microbes, are often harmful for paleolithic paintings, because they are related to
constructive (mineral precipitation) and destructive (substrate dissolution) processes affecting different substrates (host-rock, speleothems, paintings, etc.) (Cañaveras et al., 1999, 2001). Therefore geomicrobiological studies were carried out to establish the role that microorganisms play in the microbial-mineral interactions that occur in hypogean environments.

This was one of the reason for testing crystal production with a number of cave isolates. We tested 34 isolates in five different culture media: medium B-4, with three different carbon sources, medium M-2, and medium CT.

Medium B-4 (Boquet et al. 1973), containing either calcium, magnesium or ammonium acetate resulted only in the production of crystals when calcium acetate was used as carbon source. The production of calcite by a number of bacteria was previously reported in this medium by Boquet et al. (1973). Using the same medium, Laiz et al. (1999) found that Acinetobacter spp. produced vaterite (85%) and calcite (15%) (Figure 8). It appears that the ability to form calcium carbonate polymorphs is widely distributed among environmental actinobacteria as 19 out of 31 tested strains (61 % of the actinobacteria) produced a considerable amount of crystals in both solid and liquid media. The tested microorganisms produced either calcite alone (e.g. Brachybacterium sp.) or both vaterite and calcite (e.g. Rhodococcus sp.).

Medium M-2 containing both calcium and magnesium acetate was used for Mg-calcite formation (Gonzalez-Muñoz et al. 2000). Seven out of 34 strains produced crystals in this medium (20 % of the tested strains). These seven strains also produced calcium carbonate polymorphs in the medium B-4. Crystalline aggregates composed of thin platelets of magnesium calcites (HMC,
high magnesium calcite) in a rosette-like arrangement, resembling minute “desert-roses”, were obtained from *Rhodococcus* sp. cultures.

Euhedral crystals of struvite, a magnesium ammonium phosphate (MgNH₄PO₄·6H₂O) were found in the CT cultures (Perez-Garcia et al. 1989) of an unidentified strain which also produced calcite and Mg-calcite.

**Conclusions**

Microbial diversity in selected habitats cannot be comprehensively studied by traditional approaches. One should be aware that only a minor fraction of the existing bacteria in an ecosystem is able to grow under laboratory conditions. However, a high species diversity was observed in the studied caves. This diversity was even higher when identification methods included PCR amplification of 16S rRNA genes (16S rDNA) and community fingerprinting by denaturing gradient gel electrophoresis (DGGE). The applied approach gave insight into a great bacterial taxonomic diversity, and allowed the detection of unexpected and unknown bacteria.

Cave bacteria were adapted to grow at low temperatures. The isolated bacteria, other than sporoactinomycetes, were psychrotolerants, as most of them can grow at 5°C and all of them grow between 13 and 45°C but optimal growth temperatures occur at 28 or 38°C. No isolate had an optimum growth temperature below 20°C and, therefore, could not be considered as true psychrophile. However, they were able to use different carbon sources at low temperatures as showed Biolog GN microplate tests.
Actinobacteria in caves were involved in biomineralization processes. This could open new insights into the role that these microorganisms can play in crystal formation and in cave geochemical cycles.

Acknowledgements

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Figure legends

**Figure 1.** Distribution of microbial isolates from cave ecosystems among different bacterial groups: Actinobacteria, other Gram positive bacteria, and Gram negative bacteria.

**Figure 2.** Relative abundance of *Streptomyces* strains from Altamira Cave, Tito Bustillo Cave, and Grotta dei Cervi.

**Figure 3.** Colonies of *Nocardiopsis* sp. grown on 4% agar and four different concentrations of peptone: 0.25, 2, 5, and 10%, from left to right, top and bottom.

**Figure 4.** Comparison of bacterial groups obtained by culture-dependent (isolation) and culture-independent (16S rRNA gene sequences) methods from Altamira and Tito Bustillo caves.

**Figure 5.** Colony-forming units (CFU) per mg of sample at four incubating temperatures (5, 13, 20, and 28°C).

**Figure 6.** Changes in relative composition of fatty acids (16:1w11c and 18:1w7c) at different temperatures.

**Figure 7.** Variations in the number of carbon sources utilized by different bacterial species at three temperatures of incubation (5, 13, and
28°C). *Av*, *Arthrobacter viscosus*; *B*, *Bacillus* sp.; *Bh*, *Bacillus halodurans*; *Bm*, *Bacillus megaterium*; *K*, *Kocuria* sp.; *P*, *Pseudomonas* sp.; *Pp*, *Pseudomonas putida*; *Su*, *Sporosarcina ureae*.

**Figure 8.** Hemi-spherulite vaterite with a planar basement obtained from *Acinetobacter* sp. Bar indicates 5 μm.
### Table 1. Identified microbial genera.

<table>
<thead>
<tr>
<th>Actinobacteria</th>
<th>Gram - Bacteria</th>
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<td></td>
<td>Staphylococcus</td>
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Figure 2

- **Streptomyces spp.**
- **Other bacteria**

Sites:

- Altamira
- Tito Bustillo
- Grotta dei Cervi
Figure 3

[Images of four sets of circular patterns labeled A, B, C, D]
Figure 6

The graph shows the percentage of fatty acids 16:1 w11c and 18:1 w7c in Gram-positive (B. halodurans, Bacillus sp., B. megaterium, A. viscosus, S. ureae) and Gram-negative (Pseudomonas sp., P. putida) bacteria as a function of temperature (°C). The percentage decreases with increasing temperature, indicating a possible impact of temperature on the fatty acid composition of these bacteria.
Figure 7

Bacterial Strains

Number of Utilized Carbon Sources

- 5°C
- 13°C
- 28°C

Av, B, Bh, Bm, K, P, Pp, Su