Moonmilk deposits originate from specific bacterial communities in Altamira Cave (Spain)

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Abstract

The influence of bacterial communities on the formation of carbonate deposits such as moonmilk was investigated in Altamira Cave (Spain). The study focuses on the relationship between the bacterial communities at moonmilk deposits and those forming white colonizations, which develop sporadically throughout the cave. Using molecular fingerprinting of the metabolically active bacterial communities detected through RNA analyses, the development of white colonizations and moonmilk deposits showed similar bacterial profiles. White colonizations were able to raise the pH as a result of their metabolism (reaching in situ pH values above 8.5) which was proportional to the nutrient supply. Bacterial activity was analyzed by nanorespirometry showing higher metabolic activity from bacterial colonizations than uncolonized areas. Once carbonate deposits were formed, bacterial activity decreased drastically (down to 5.7% of the white colonization activity). This study reports on a specific type of bacterial community leading to moonmilk deposit formation in a cave environment as a result of bacterial metabolism. The consequence of this process is a macroscopic phenomenon of visible carbonate depositions and accumulation in cave environments.
Introduction

Microorganisms are responsible for the decomposition of organic matter and the biogeochemical cycling of elements in soils and other environments. These processes involve a huge diversity of microorganisms [14] representing highly complex phenomena. Microorganisms have been an active factor shaping our environment and mineral depositions in caves are examples of this activity [3, 4]. Carbonate depositions and dissolution are common processes in caves [10, 26, 32] and the participation of microorganisms in these processes has been reported [2, 9]. One type of carbonate precipitates attracting attention are the moonmilk deposits.

Moonmilk refers to a variety of microcrystalline mineral aggregates ranging from soft and wet to a dry and powdery appearance [9, 26]. It is usually composed of calcite, aragonite, or hydromagnesite although can be formed by other carbonate and noncarbonate minerals [26]. Several mechanisms, biotic and abiotic, have been proposed for the formation of moonmilk [26]. The participation of microbial activity has been suggested by a number of authors although there is confusion on the type of microorganisms and their role in moonmilk formation. Some studies suggested fungi as the major participants in the process [7] although more recent investigations proposed bacteria as the major inducers of carbonate deposition forming moonmilk in caves [2, 9]. Besides, the existence of complex microbial communities thriving in cave environments has been highlighted [2, 9, 19, 28, 30, 31] indicating that the process of carbonate deposition could be a consequence of the activity of diverse microbial communities.

In carbonate caves, precipitation of carbonates usually occurs at pH values of 8 and above [6, 23] since the equilibrium CO₂-carbonate is strongly dependent on pH,
assuming other critical factors are met, such as an elevated partial pressure of CO$_2$, and
elevated concentrations of carbonates and calcium ions [6, 15, 21]. Microorganisms or
their communities can alter their microenvironment as a result of microbial metabolism
[3, 28]. If products of microbial activity result in a pH increase in the environment,
precipitation of calcium carbonate can occur in caves [2, 4, 32]. The role of microbial
communities and the regulation of microbial activity previous to and during carbonate
deposition remain to be investigated.

The aim of this study is to describe the structure and activity of the microbial
communities leading to the formation of moonmilk deposits which is important to
determine the origin and fate of microorganisms in relationship to the formation of
carbonate deposits in caves.

**Materials and Methods**

**Study site**

This study was carried out in Altamira Cave (Cantabria, Northern Spain)(Fig. 1). Its
unique polychromic paintings dated about 15,000 years-old from the Paleolithic. A
description of Altamira Cave, its archaeology and paintings has been previously
published [22] as well as its environmental parameters [8, 34] (Fig. 1). Temperature is
around 13-14°C and relative humidity is near saturation. Bacterial communities in white
[31], yellow and grey [30] colonizations have also been reported in Altamira Cave and
they have been microscopically documented in recent publications [13] as well as the
moonmilk deposits [8, 9].
Sample collection and processing

Areas covered with moonmilk, zones lacking moonmilk depositions and visible microbial colonizations, and white colonizations were sampled. Four samples from each area were collected and processed from the Hall of the Walls (Figure 1). About 50 mg samples were collected using sterile scalpels. The portion of samples aimed for RNA-based analyses were immediately preserved in RNAlater (Ambion, Austin, TX, USA) and then placed on ice until arrival to the laboratory where they were stored at -80ºC until processed. Portions of samples for pH determinations and nanorespirometry were analyzed in situ and those for culture and growth were preserved on ice until arrival to the laboratory where they were immediately processed.

RNA extraction and PCR amplification

RNA-based molecular surveys have been proposed as the procedure to identify the major members of metabolically active bacterial communities because RNA content per cell is proportional to microbial activity and growth [30, 31]. Total RNA was extracted using the RNAqueous4PCR kit (Ambion Inc., Austin, TX, USA) according to the manufacturer’s recommendations. The protocol of total RNA extraction included DNaseI treatment to remove any DNA remaining in the final extract. A reverse transcriptase reaction was carried out from the total extracted RNA using Thermoscript (Invitrogen, Carlsbad, CA, USA). The 16S rRNA gene-specific primer 518R (5’-ATT ACC GCG GCT GCT GG) [24] was utilized during reverse transcription at a temperature of 55ºC for 1 h. Controls lacking reverse transcriptase were carried out to assess the potential presence of DNA in extracted RNA and always resulted in negative
amplifications. Amplification of 16S rRNA gene fragments from cDNA was performed by PCR to obtain bacterial community fingerprints and to construct 16S rRNA gene libraries as described below. The DNA polymerase ExTaq (Takara, Shiga, Japan) was used throughout this study. Thermal conditions for the amplification reaction consisted on the following steps: 95ºC for 2 min; 30 cycles of 95ºC for 15 s, 55ºC for 15 s and 72ºC for 1 min; and a final incubation at 72ºC for 10 min. Amplifications were performed in a BioRad iCycler iQ thermal cycler (BioRad, Hercules, CA).

Microbial community fingerprints

Each bacterial community can be characterized by unique molecular fingerprints representing the most abundant bacterial members of the community [16, 24]. These DNA banding patterns allow community comparisons [24] as described below. Microbial community fingerprints were obtained by DGGE analysis. PCR amplification was performed using cDNA as template and the primer pair 341F-GC (5’-CC TAC GGG AGG CAG CAG; with a GC-rich tail added at its 5’-end) and 518R following a previously described procedure [24] with the modifications introduced by Portillo and Gonzalez [27]. According to these modifications, product amplification occurred only during the exponential phase of PCR allowing a relative quantification of banding patterns. Gels obtained by DGGE were digitalized using Kodak 1D image analysis software (Kodak, New Haven, CT). Images were analyzed with the program tnimage (http://entropy.brneurosci.org/tnimage.html) using its densitometry function. Relative quantification of bands from each bacterial community profile was performed according to Portillo and Gonzalez [27]. Comparisons between community fingerprints were performed as described by Portillo and Gonzalez [27] using the software fingshuf.
which calculates a Cramér-von Mises-type statistic through a Monte-Carlo test procedure to determine the significance of differences between microbial community fingerprints from different samples.

**Bacterial 16S rRNA gene libraries**

Gene libraries were used for clone sequencing and identification of the major members of the bacterial communities under study. The 16S rRNA libraries were constructed from samples of moonmilk and white colonizations. To construct these libraries the primer pair 27bF (5’-AGA GTT TGA TYM TGG CTC AG) [35] and 518R was used. PCR products were purified using the JetQuick PCR Purification Spin Kit (Genomed, Löhne, Germany) and cloned with the TOPO TA Cloning Kit (Invitrogen, Carlsbad, CA). Clone screening was carried out by Denaturing Gradient Gel Electrophoresis (DGGE; see above) as previously described [17] selecting the clones corresponding to the major DNA bands. Plasmids were purified using the JetQuick Plasmid Purification Spin Kit (Genomed, Löhne, Germany) and sequenced by SSAD Sequencing Services (CSIC, Madrid, Spain). In order to determine the grade of coverage of the detected sequences with respect to the bacterial communities, accumulation curves were constructed according to Hughes and Hellmann [20].

**Nucleotide sequence analysis**

Sequence data were edited using the software Chromas, version 1.45 (Technelysium, Tewantin, Australia). Homology searches from the nucleic acid sequences were performed using the Blast algorithm [1] at the NCBI (National Center for
Biotechnology Information; http://www.ncbi.nlm.nih.gov/Blast/). Sequences were checked for chimeras using the program Ccode as described by Gonzalez et al. [18].

**pH measurements and pH increase during growth**

The pH in the center of white colonizations was measured using pH microelectrodes (Unisense, Århus, Denmark) attached to a micromanipulator following previously described procedures [33].

The ability of the bacterial community from white colonizations to raise the pH as a result of their metabolism was assessed by inoculating nutrient broth at different concentrations with freshly collected white colonizations. Flasks were incubated at 20°C for two to three days. Nutrient broth at standard concentration, and at 1/10, 1/100, and 1/1000 dilutions of the recommended concentration were used in this study. The pH was monitored during bacterial growth. Aliquots were collected over time and measurements of pH were performed with a standard desktop pH meter.

**Nanorespirometry**

The aerobic respiratory activity by the bacterial communities in white colonizations, moonmilk, and areas lacking visible colonization was assessed by nanorespirometry. The procedure has been described in detail by Nielsen et al. [25]. Briefly, each sample was placed in the bottom of a preweighted glass cuvette (3 mm inner diameter) and filled with 100 µl sterile distilled water. As glass is completely impermeable to oxygen, oxygen was only supplied by molecular diffusion from the atmosphere into the water column above the sample in the glass well. Oxygen consumption by the sample was
monitored using an oxygen microelectrode with a sensor tip diameter of 20 µm (Unisense, Århus, Denmark) and determining the concentration gradient through the water column in the cuvette. The sensor was attached to a motorized micromanipulator system controlled by computer software (Unisense). At about two hours, the gradient reached a steady state for our experiments and the depth profile of oxygen concentration followed a linear gradient [25]. Controls lacking sample showed constant oxygen concentration along the depth profile. The slope of oxygen concentration versus cuvette depth was used to provide relative estimates of oxygen consumption [25] by respiratory activity in the sample and the oxygen flux towards the bottom of the cuvette [12]. After these measurements, samples were dried (105°C for 24 h) and weighted. Slopes were normalized by sample dry weight.

Results

Figure 1 shows examples of white colonizations and moonmilk deposits studied in Altamira Cave. DGGE community fingerprints from white colonizations and moonmilk deposits were compared (Fig. 2) in order to assess if these microbial communities showed similarities. No differences among moonmilk samples processed independently were detected in the distribution of the banding pattern through DGGE (Fig. 3). A similar analysis with samples collected from white colonization has been previously shown [31]. Comparison of molecular profiles from moonmilk deposits and white colonizations using fingshuf [27] showed that the major representants of the metabolically active bacterial community in moonmilk deposits represented a subset of the active community in white colonizations. According to the description of this type of analyses provided by Portillo and Gonzalez [27], this implies that moonmilk
fingerprints are included in the community profile of white colonizations. Most of the major components of the microbial community from moonmilk deposits observed from DGGE analysis were present in the profile from white colonizations as seen in Fig. 2. The inverse comparison showed significant differences (P<0.01) indicating that the banding pattern from metabolically active bacteria in white colonizations were not fully integrated in the active communities of moonmilk depositions.

The major members of the metabolically active bacterial community were identified (Table 1) through cloning, sequencing, and comparison by DGGE analysis. A total of 82 and 85 clones were analyzed for moonmilk and white colonizations, respectively. Alphaproteobacteria, mainly characterized by the genera *Sphingomonas* and *Methyllobacterium*, were the major components in moonmilk deposits, followed by Gammaproteobacteria (i.e., *Pseudomonas*), Betaproteobacteria (i.e., *Aquabacterium*), and Actinobacteria (represented mainly by the genera *Pseudonocardia* and *Propionibacterium*). The proportion of each phylotype (based on band intensity from DGGE analysis) in white colonizations and moonmilk is presented in Table 1. Similar composition of the bacterial community has been described by Portillo et al. [31] in white colonizations. A comparison of the cumulative curves obtained during the analyses of clones from 16S rRNA libraries of moonmilk and white colonization samples is presented in Fig. 4 showing similarity in the number of detected taxa and in the trend to level-off of the curves indicating similar levels of diversity in these samples.

To examine the capability of the bacteria forming white colonizations to induce carbonate precipitation, these colonizations were collected and inoculated in media with different nutrient concentrations. The bacterial assemblages lead to progressive increases of pH up to values above pH 8 during growth. Generally, the highest nutrient concentrations induced the highest pH values suggesting that the level of pH increase is
dependent on the availability of organic nutrients (Fig. 5). Relatively poor nutrient media (i.e., NB diluted 1/1000) also induced pH raising reaching levels slightly above pH 8. These results represent evidence suggesting that the heterotrophic bacterial metabolism in white colonizations is able to induce a pH increase to a level high enough to produce carbonate precipitation.

*In situ* pH determinations using microsensors were performed directly in white colonizations and resulted in pH values ranging from pH 8.7 to pH 9.5. These data also confirmed that bacteria in white colonizations are able to induce a pH above 8 which is generally required for calcium carbonate precipitation in the cave environments.

In order to analyze the effect of bacterial activity on moonmilk formation as well as the effect of carbonate precipitation on bacteria, the relative respiratory activity of samples from moonmilk, white colonizations, and uncolonized samples was estimated. Results showed that white colonizations presented a much higher (P<0.001, ANOVA) aerobic respiratory activity than uncolonized samples (59.5% of white colonizations activity) (Fig. 6) suggesting an increase in activity due to a massive development of bacteria forming white colonies. Besides, moonmilk samples showed a significantly reduced (P<0.001, ANOVA) aerobic respiratory activity (5.7% of white colonization activity) when compared to colonized and areas showing no visible colonization (Fig. 6).

**Discussion**

Microbial communities influence carbonate precipitation in cave environments [2, 9] although the details of this interactions remain to be described. As an example, Cañaveras et al. [9] proposed that bacteria and not fungi were involved in the process of
Barton and Northup [2] pointed out that carbonate precipitates could lead to entombment of bacteria which get entrapped in these crystals precluding them to grow and reach appropriate nutrients. Portillo et al. [30, 31] have shown the existence of complex and differential microbial communities leading to visually differentiated colonizations of distinct colors on cave walls. In this study, an analysis of bacterial communities constituting white colonizations provides evidence supporting the origin of moonmilk deposits on previous bacterial colonizations. The bacterial communities constituting white colonizations included the major metabolically active components present in bacterial communities from moonmilk deposits.

Different types of colonizations have been reported in Altamira Cave [13]. These colonies have been distinguished based on its coloration as white, yellow and grey and present distinct bacterial communities [30, 31]. While the metabolically active bacterial community present in moonmilk deposits is integrated in the active community found in white colonizations, the community fingerprints from other types of colonizations in Altamira Cave showed significant differences with the moonmilk community of metabolically active bacteria (P<0.001) as well as with white colonizations [31]. Soil and wall samples have also been analyzed through DGGE analyses [28] and resulted in significantly different profiles when compared to moonmilk fingerprints. Since community fingerprints are highly specific for each type of sample [24, 27], the existence of similar profiles among two different types of samples represent a strong indicator of a relationship between these communities. In the studied case, previous conclusions [9] supporting the bacterial origin of moonmilk depositions and the results herein presented constitute solid evidence suggesting that the bacterial communities inducing moonmilk deposits originate through massive bacterial
development in Altamira Cave and these colonizations are those forming white
colonies.

White colonizations in Altamira Cave show pH values above pH 8.7 which
support their capability to induce carbonate precipitation. Growth experiments present
evidence supporting that this elevated pH could be the result of bacterial metabolism. In
these experiments, we have observed that pH increase is related to nutrient availability
even at organic nutrient concentrations equivalent to actual cave levels (Fig. 5) [32].

Bacterial growth in caves is usually a result of organic inputs [2, 11, 32] and different
types of colonies described on cave walls have been shown to induce distinctive values
of pH, from acidic to alkaline pH [29]. Bacterial growth, in the studied case, resulted in
white colonizations which were mainly constituted by heterotrophic bacteria.

Progressive and slow growth can lead to the formation of white colonies, this
development of bacteria could produce pH increases in these colonies which could
induce carbonate precipitation under cave conditions.

An increase in bacterial activity is observed during the development of bacterial
colonizations in caves. In Altamira Cave, white colonizations showed much higher (2
fold) aerobic respiratory activity than zones lacking visible bacterial accumulation.

Also, bacterial respiratory activity was reduced (to 5.7% of the white colonization
activity) during carbonate precipitation leading to moonmilk deposits. The result of
massive growth and bacterial activity is a progressive increase in pH as a result of
organic nutrient processing through aerobic heterotrophic metabolism. Thus, bacterial
communities forming white colonies generated a microenvironment with high pH which
induces carbonate precipitation under cave conditions [2, 9, 34]. Progressive calcium
carbonate precipitation results in macroscopic calcitic moonmilk deposits [9]. These
formations constitute the moonmilk deposits. As a consequence of this progressive
accumulation of calcite, microorganisms get trapped in the crystal matrix where the availability of organic nutrients is reduced [2]. Thus, bacterial activity progressively decreases during calcification reaching close to undetectable levels of respiration.

This study presents details on the process and consequences of bacterial activity, growth, community structure and contribution to moonmilk formation, the origin of moonmilk depositions, and the role of bacteria on shaping cave environments. This information also contributes to understand the role of microorganisms in trapping inorganic carbon in underground systems [4, 5, 21, 32].

Acknowledgements

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References


Table 1. Identification and proportion of the major metabolically active components of the bacterial communities in white colonizations and moonmilk deposits from Altamira Cave. Migration distance corresponds to those in Fig. 2.

<table>
<thead>
<tr>
<th>Migration</th>
<th>Accession No.</th>
<th>Taxonomic affiliation</th>
<th>White</th>
<th>Moonmilk</th>
</tr>
</thead>
<tbody>
<tr>
<td>127</td>
<td>EF188810</td>
<td><em>Pseudomonas</em> (Gammaproteobacteria)</td>
<td>11.1</td>
<td>14.8</td>
</tr>
<tr>
<td>237</td>
<td>EF188436</td>
<td><em>Moraxella</em> (Gammaproteobacteria)</td>
<td>2.5</td>
<td>2.4</td>
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<tr>
<td>323</td>
<td>EF188431</td>
<td><em>Taxeobacter</em> (Bacteroidetes)</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>EF188798</td>
<td><em>Escherichia</em> (Gammaproteobacteria)</td>
<td>4.1</td>
<td>3.1</td>
</tr>
<tr>
<td>350</td>
<td>EF188320</td>
<td><em>Sphingomonas</em> (Alphaproteobacteria)</td>
<td>16.7</td>
<td>16.9</td>
</tr>
<tr>
<td>385</td>
<td>EF188428</td>
<td><em>Hyphomicrobium</em> (Alphaproteobacteria)</td>
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<td>1.1</td>
</tr>
<tr>
<td>407</td>
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<td><em>Aquabacterium</em> (Betaproteobacteria)</td>
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<td>15.2</td>
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<td></td>
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<td>1.3</td>
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<td>757</td>
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<td><em>Propionibacterium</em> (Actinobacteria)</td>
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</table>

1 DGGE bands are defined by electrophoretic migration distance (arbitrary units).

2 Percentage of each identified DGGE band with respect to total density.
Figure legends

Figure 1. Location of Altamira Cave (A) and a partial map of this cave (B) showing the Hall of the Walls were samples were collected. Examples of white colonizations (C) and moonmilk (D) are also shown. Bars in C and D represent 1 cm.

Figure 2. Molecular fingerprints of the metabolically active bacterial communities in white colonizations (A) and moonmilk deposits (B) from Altamira Cave. These fingerprints were obtained by DGGE and were based on RNA analyses. Migration distances indicated on the left correspond to those reported in Table 1.

Figure 3. Comparison of microbial community fingerprints of the metabolically active bacteria at Altamira Cave from three samples of moonmilk independently processed. Identical molecular profiles were obtained.

Figure 4. Cumulative curves of processed clones and number of detected taxa for 16S rRNA gene libraries constructed from white colonizations (filled squares) and moonmilk deposits (open squares). The 1:1 line is shown for reference.

Figure 5. Measurements of final pH reached during growth of the microbial communities from white colonizations collected in Altamira Cave as a function of nutrient concentration. Different nutrient concentrations were
Figure 6. Percentage of oxygen consumption estimated by nanorespirometry in samples of white colonizations (considered as 100%), moonmilk deposits, and areas showing no visible colonization at Altamira Cave. Error bars indicate standard deviation from four analyses.
Figure 1

A

Spain

France

Portugal

Altamira Cave

Entrance

Kitchen Hall

Polychromes Hall

Hall of the Walls

B

C

D

N

0 5 10 20 m
Figure 5