

THE SPANISH BIODETERIORATION AND BIODEGRADATION GROUP

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The *Biodeterioration and Biodegradation Group* (<http://www.semicro.es/biodet/presentacion.htm>) was founded in 1989 within the Spanish Society for Microbiology (SEM) (<http://www.semicro.es/>).

Among Group's activities are organization of Biodeterioration specialized meetings, monographic round tables on up-to-date, specific subjects, doctorate and postgraduated courses on "*Materials Biodeterioration*", in which students receive a wide and in-deep training on the biodeterioration processes that materials suffer in different environments.

In 2005 on behalf of the *International Biodeterioration and Biodegradation Society* (<http://www.biodeterioration.org/>) the Spanish Biodeterioration and Biodegradation Group organized, in Madrid (Spain), the *13th International Biodeterioration and Biodegradation Symposium* (<http://www.ibbs-13.org>). Plenary sessions were published in a monographic issue of *International Microbiology* (2005, Vol. 8, No. 43, pp. 153-230) (<http://www.im.microbios.org/0803/0803%20contents.pdf>). Additionally, some of works presented were selected for publication in a special issue of *International Biodeterioration and Biodegradation* (2006, Vol. 58, No. 3-4, pp. 107-260). (http://www.sciencedirect.com/science?_ob=PublicationURL&_cdi=6030&_pubType=J&_acct=C000047350&_version=1&_urlVersion=0&_userid=885385&md5=2e32811af2cb24dae43e5ec284981211&jchunk=58#58).

In both journals, papers about Cultural Heritage Biodeterioration were published.

Coinciding with the XXI SEM Meeting, held in Seville in September 2007, a round table on "Biodeterioration and Biotechnology of

Cultural Heritage" was organized. The four communications discussed are published in this current issue of Coalition.

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BACTERIAL DIVERSITY IN THE CAVE OF ALTAMIRA

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The study of microorganisms involved in deterioration of prehistoric paintings is of great importance to preserve these singular samples of cultural heritage. This study presents, as an example, results from a research carried out in Altamira Cave (Cantabria, Spain). Altamira Cave contains numerous and well known paintings from the Upper Palaeolithic, dated back around 15,000 years. This work focused in understanding the microorganisms inhabiting this singular system and the potential consequences of its microbial diversity for conservation of these valuable paintings.

Introduction

The importance of microorganisms in cultural heritage have been generally overlooked because either the methods used were not appropriated for the proposed objectives or their potential implications in cave conservation was not considered. In environmental microbiology, using the appropriate methodology is of critical importance. Classical microbiological methods involve the use of culturing techniques for the detection of microorganisms. Culturing implies a need to grow up a microorganism in the laboratory to be detected. Since the growth conditions of most microorganisms in our planet are still unknown, we can only culture up to 1% of the microorganisms present in any given sample (Ward et al. 1990, Gonzalez and Saiz-Jimenez 2004). Microscopy observation, for instance, is unable to provide microbial identification due to the relatively

low morphological diversity with respect to the known specific diversity of microorganisms.

In recent years, a novel methodology is being developed to approach the detection of environmental microorganisms independently of a need to culture them. The use of molecular techniques based on the analysis of nucleic acids is transforming the way in which environmental microbiology, and the general microbiology, is being looked up. Today, it is known that the microbial diversity in our planet is huge, well beyond what can be experimentally determined (Curtis et al. 2002). These molecular methods allow to detect microorganisms without a need for culture them and so the researchers can get a vision of the actual microbial community *in situ*, without modifications due to microbial growth in the laboratory.

Studies available so far report on the isolation and analysis of a very reduced number of

microbial groups. For instance, some heterotrophic bacteria and fungi are often cultured and are generally relatively easy to grow up in the laboratory. However, most physiological groups of microorganisms have never, or rarely, being grown up from cultural heritage samples. Examples, shown in Table 1, include most anaerobic bacteria such as the sulfate-reducing bacteria, denitrifying bacteria, or methanogenic archaea, lithotrophic microorganisms (both aerobic and anaerobic ones), and many others. An example of frequently cultured microorganisms are the phototrophs. Microalgae and Cyanobacteria have been reported to grow on historic sites, generally leading to green-colored films able to cover large surfaces of murals, paintings, or walls of a variety of monuments. Some phototrophs able to grow up at reduced light intensities have been often reported to develop in caves with permanent illumination installed (Albertano and Urzi 1999).

Table 1. Some physiological groups of microorganisms classified by their expected relative abundance in caves with prehistoric paintings, the risk involved in attempting to culture them, the frequency of reports for these cultures, and some common examples

Type of metabolism	Oxygen tolerance	Relative abundance	Frequency of cultivation	Basic examples
Heterotrophic	Aerobic	High	High	Many Bacteria, Fungi
Heterotrophic	Anaerobic	High	Low	<i>Clostridium</i>
Lithotrophic	Anaerobic	Low/Medium	Very low	<i>Thiovulum</i>
Lithotrophic	Aerobic	Low	Very low	<i>Thiobacillus</i>
Sulfate-reducers	Anaerobic	Medium	Very low	<i>Desulfovibrio</i>
Methanogens	Anaerobic	Low	Very low	<i>Methanosarcina</i>
Denitrifiers	Anaerobic or Aerobic	Low/Medium	Very low	<i>Pseudomonas denitrificans</i>
Acetogens	Anaerobic or Aerobic	Low/Medium	Very low	<i>Acetobacterium</i>
Fe-reducers	Anaerobic	Low	Very low	<i>Shewanella</i>
Phototrophic	Aerobic	Low to High	High	Cyanobacteria, microalgae
Unknown	Unknown	Low	Never	Candidate Divisions

Methodology

A case study carried out in Altamira Cave (Cantabria, Spain) is presented here. The methodology used during this study has been previously described (Gonzalez et al. 2003, Gonzalez and Saiz-Jimenez 2004, Portillo et al. 2008). Briefly, DNA and RNA were extracted from minute samples. Analysis based on DNA allow to detect the

microorganisms present in a sample, while those performed based on RNA provide with information on those showing significant metabolical activity within their community. Bacterial and archaeal 16S rRNA gene fragments were amplified by PCR (Polymerase Chain Reaction) and processed for DNA library construction with the aim of sequencing and microbial community fingerprints by DGGE

(Denaturing Gradient Gel Electrophoresis). Bioinformatic analyses were performed in order to obtain the taxonomic affiliation of the retrieved sequences which was compared with molecular profiles of these communities obtained by DGGE.

Results and Discussion

Reports from the culture of microorganisms from samples collected in Altamira Cave showed that three major groups represented practically the total microbial community in this cave (Laiz et al. 2003). These groups were the Actinobacteria (mostly *Streptomyces*), Proteobacteria (mostly Gammaproteobacteria), and Firmicutes (mostly *Bacillus* spp.). Recently, molecular analysis have shown the presence of a large number of different microorganisms involved completely different physiologies (Schabereiter-Gurtner et al. 2002, Gonzalez and Saiz-Jimenez 2005, Portillo et al. 2008). A preliminary study on this topic was presented by Schabereiter-Gurtner et al. (2002) showing the presence of several microbial groups, never detected before in Altamira Cave; these groups were Acidobacteria, Bacteroidetes, Planctomycetes, and Chloroflexi. These groups represented about 45% of the detected microorganisms in that study. In this study of Schabereiter-Gurtner et al. (2002), Proteobacteria represented 50% of the total studied community. Actinobacteria appeared to represent just about 5% of total in contrast to the great abundance found by culturing procedures. Firmicutes, relatively abundant by

culturing methods, was not even present in this preliminary study (Schabereiter-Gurtner et al. 2002). The differences generated by the different methods used in the analyses were obvious and highly significative, leading to a change of the perspective related to the conservation of the cave.

More recent and intense surveys of the microbial communities in Altamira Cave have shown the presence of a much large complexity in these communities (Gonzalez et al. 2008). For instance, the group Proteobacteria has been the most frequently detected although it includes representatives from the Alpha, Beta, Delta, and Gamma Proteobacteria. The Alpha and Gammaproteobacteria were the most commonly found. The major components of the microbial communities in Altamira Cave had been identified and were represented by bacteria related to the genus *Sphingomonas* (Alphaproteobacteria), Acidobacteria, Gammaproteobacteria (Enterobacteriales and Pseudomonadales), Deltaproteobacteria (Desulfovibrionales), Betaproteobacteria (Burkholderiales and Rhodocyclales), and Actinobacteria (Actinomycetales). The Actinobacteria represent a low fraction of the total, generally around 5% of total sequences which is in agreement to the results of Schabereiter-Gurtner et al. (2002). From our results, the Firmicutes were also detected using molecular methods although they constitute a low percentage of the bacterial community.

Table 2. Some of the microbial groups rarely or never reported before as important components of the microbial communities in Altamira Cave. Estimate of their diversity, current knowledge of their status, and the level of knowledge on their metabolism are reported.

Microbial group	Status of detection	Abundance	Physiology and role
Sulfate-reducing bacteria	Active/ mostly uncultured	Very high	Known
Low-temperature Crenarchaeota	Active/uncultured	Very high	Mostly unknown
Acidobacteria	Active/uncultured	Very high	Barely known and highly diverse
Bacteroidetes	Active/uncultured	Medium/high	Mostly unknown and highly diverse
Planctomycetes	Active/uncultured	Medium/high	Mostly unknown and highly diverse
Chloroflexi	Uncultured	Medium	Mostly unknown
Verrucomicrobia	Active/uncultured	Medium	Mostly unknown
Nitrospirae	Uncultured	Medium	Mostly known

Interestingly, besides the microorganisms frequently reported, a number of microbial groups have never, or sporadically, been reported in this cave (Table 2), even if they constitute a highly significant fraction of the total community. For instance, within the Proteobacteria, the sulfate-reducing bacteria belonging to the Deltaproteobacteria, have never been reported previously and we have detected their presence as metabolically active

microorganisms (by RNA analysis). Previous finding on sulfate reducing bacteria have been rarely mentioned. Sulfate reducing bacteria could have important consequences for pigment conservation as previously reported (Portillo et al. 2006). The Acidobacteria have been extensively studied by Zimmermann et al. (2005) although their metabolism and role in the studied cave remains to be investigated.

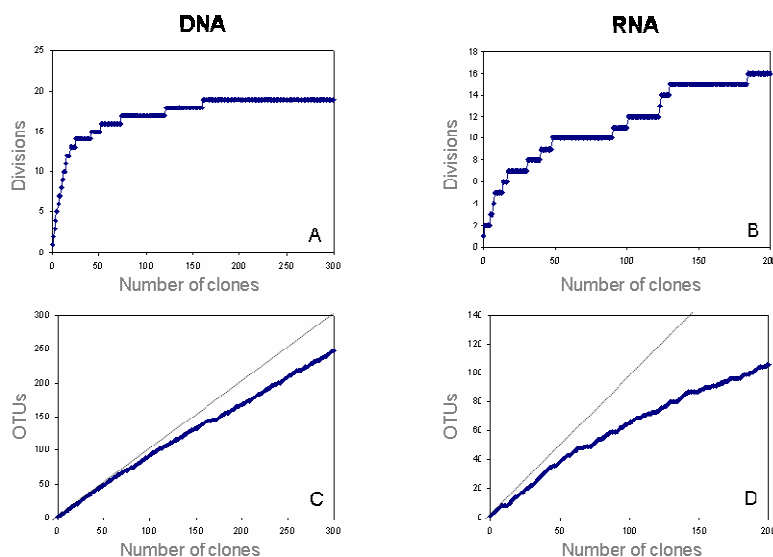


Figure 1. Cumulative curves of the number of processed sequences retrieved from Altamira Cave and the number of bacterial divisions (A and B) or different microorganisms (shown as OTUs or Operational Taxonomic Units) (C and D) analyzed.

The Archaea have been never reported in Altamira Cave until the study by Gonzalez et al. (2006). Specifically, the low-temperature Crenarchaeota represent a highly diverse and ubiquitous microbial group in Altamira Cave although their role, threaten to the paintings and metabolism is still unknown due to the difficulty in culturing these microorganisms. Other microbial groups barely known and present in Altamira Cave as metabolically active members of the communities and in a significant proportion of these microbial communities are reported in Table 2. The metabolism of most of these groups is unknown and so, their potential risk for the conservation of the cave is pending of further developments in microbiology.

These last studies that we are carrying out suggest that the actual diversity in Altamira Cave is much higher than shown in the results obtained so far. Figure 1 shows cumulative curves of sequencing results obtained in Altamira Cave during the campaigns carried

out in the last years. We have been able to identify the bacterial groups (i.e., at the Division level) present in the cave as determined from molecular analysis based on DNA. When this same analysis is performed on the different microorganisms present in Altamira Cave, we detect that the cumulative curve barely experiments a downwards curvature. This result clearly indicates that the actual number of different microorganisms in Altamira Cave is huge, and much higher than previously imagined, even if those microorganisms belong to already detected bacterial divisions. When the analysis is performed using molecular techniques based on RNA, that is, on those microorganisms showing considerable metabolic activity within the microbial community, then we observe that the number of bacterial divisions detected in the studied cave is similar to that determined from DNA; this suggests that most of the bacterial divisions detected in this cave somehow have representatives with considerable activity. However, when

analyzing the results of the different microorganisms detected in Altamira Cave using RNA-based techniques, we observed that the cumulative curve based on different metabolically active microorganisms levels off significantly suggesting that the number of metabolically active microorganisms from Altamira Cave are not as abundant as those present. Consequently, Altamira Cave presents a huge number of different microorganisms although only a fraction of them are actively participating in microbial development or growth in the cave.

While microbiologists know well that environmental conditions directly influence the development of specific microorganisms, and that a high microbial diversity is present in this cave, unpredictable changes in these microbial communities could potentially occur if the environmental conditions of the cave change. Thus, one of the preliminary objectives in the conservation of caves with prehistoric paintings should be to maintain them in the closest conditions to those that have maintained these caves for thousands of years (Allemand and Bahn, 2005). Mass tourism, illumination set ups, and other modifications to the cave environmental conditions could result in unpredicted changes in the microbial communities, likely to enhance the growth of microorganisms that never had any significance in this cave.

The study of microbial communities in Altamira Cave have revealed the presence of metabolically active microorganisms rarely mentioned in previous studies. Examples are the Crenarchaeota (Archaea), the Acidobacteria and several other bacterial groups (Table 2). These groups represent a significant fraction of the microbial communities. This and the fact that the role of these microbial communities is poorly understood would represent a risk for the conservation of this UNESCO World Heritage site.

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