WHAT CAN MOLECULAR MICROBIOLOGY TELL US ABOUT LASCAUX CAVE?

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Lascaux Cave contains paintings from the Upper Paleolithic period. Shortly after discovery in 1940, the cave was seriously disturbed due to major destructive interventions (e.g. excavations, air conditioning, artificial light installation, etc.). In 1963, the cave was closed due to algal growth on the walls. In 2000 the air conditioning system was replaced. In 2001, (a few months after that replacement), the vault, walls, sediments and soil were colonized by *Fusarium solani*. Later, black stains also appeared at the entrance, which extended in 2007 to the rest of the cave . Between 2001 and 2004, and again in 2008, the cave was treated with benzalkonium chloride. Recently, we have studied the microbial communitie in Lascaux Cave using molecular tools. We conclude that the indigenous bacterial community was replaced by microbial populations selected for by biocide application and other major destructive interventions. In addition to the microbial populations, entomopathogenic fungi play an important role in the cave and arthropods contribute to the dispersion of spores and fungal development.

1. Introduction

The conservation of Paleolithic paintings in caves is of great interest because they represent a priceless cultural heritage of all humankind. Among the Paleolithic paintings, those at Chauvet and Lascaux, France, and at Altamira in Spain, show remarkable sophistication. In recent times, some of the most important caves are suffering episodes of biological contamination that might damage the paintings (SCHABEREITER-GURTNER et al., 2002; DUPONT et al., 2007)

The Cave of Lascaux was discovered in 1940. The importance of its paintings was recognized shortly after its discovery and they are considered to be among the finest rock art paintings. As soon as it was open to the public the cave attracted many visitors, which amounted up to 1,800 every day in the 1960's (SIRE, 2006). This seriously disturbed the cave microclimate and had a strong impact on the whole cave.

Rock art tourism started at the beginning of the past century. At that time, no scientific knowledge on conservation problems existed, therefore management decisions adopted often resulted in great harm for the future of many caves. In the Cave of Lascaux, the conditions for visits between 1945 and 1948 and in 1958 (and the impact of massive tourism thereafter) were two of the main problems. In fact, lighting which began in 1960 caused the growth of a green biofilm on the wall paintings, initially identified as produced by *Chlorobotrys*, a Xanthophyta alga. Years later, the observation of zoospore formation in one of the algal isolates, not previously detected, led to its proper specific determination as *Bracteacoccus minor*, a member of the Chlorophyta (LEFÉVRE, 1974). This green biofilm, considered as "la maladie verte", was the first one of the different contamination episodes suffered by the cave leading to its closure in 1963 due to the damage produced by visitors, lighting, and algal growth on the paintings.

SIRE (2006), in an excellent historical report on Lascaux management, stated that the treatments for defeating "la maladie verte" included a combined spray application of streptomycin and penicillin for bacteria and a subsequent treatment with formaldehyde for algae. These applications were effective until 1969 when it was necessary to start again and a programme of periodic maintenance and cleaning was adopted.

Between July and September 2001 the first evidence of a fungal outbreak appeared along with an associated bacterium *Pseudomonas fluorescens* (ALLEMAND, 2003; ORIAL AND MERTZ, 2006). In August 2001, treatment of the outbreak began but its rapid expansion led to a more intensive treatment in September using alkyl dimethylbenzylammonium chloride solutions plus streptomycin and polymyxin. The soils were treated with quicklime (SIRE, 2006). In 2004 benzalkonium chloride treatments were replaced by mechanical cleaning and air extraction and recovery of cleaning debris. At present there is strict control on access to the cave, and all interventions are recorded and discussed at the "Comité Scientifique International de la Grotte de Lascaux", created in 2002 by the French Minister for Culture and Communication.

In 2007, black stains (Fig. 1) were found in the vault and passage banks. Although their origin is unknown, dematiaceous hyphomycetes, producing olive-green to black colonies, were isolated from the stains (BASTIAN AND ALABOUVETTE, 2009).

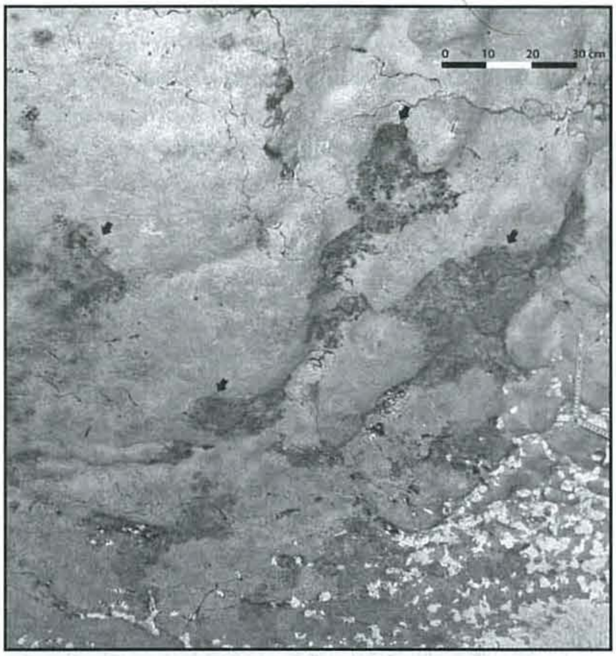

Figure 1: Black stains in the vault of the Passage, June 2008. Picture protected by copyright: Ministère de la Culture et de la Communication, DRAC d'Aquitaine and Centre National de la Préhistoire.

In the last year a debate was initiated in European and U.S. media on Lascaux black stains (e.g. DE ROUX, 2007; SIMONS, 2007; FOX, 2008). In addition to a historical description on the works carried out in the cave since the discovery, comments on the problem from different experts were summarized and the appearance of black stains noticed.

2. Cave Ecology

Why has Lascaux Cave suffered succesive biological invasions since its discovery? The problem derives from the public interest and pressure of rock art tourism and the erroneous conception that all rock art should be exposed to public contemplation. This concept is in general opposition to effective conservation of the rock art. This is because opening a cave immediately results in a sudden change of microclimate with accompanying deterioration of speleothems and rock art paintings.

Opening a cave also impacts cave biology. Bacteria, fungi, and arthropods, all have constructed delicate and balanced trophic relationships between predator and prey and the strength of interactions between species can be interrupted by tourism and cave preparation for tourism which include excavations and major destructive interventions.

The situation in Lascaux is particularly worrying because the cave has had two different fungal invasions in six years. In 2001 the presence of members of the *Fusarium solani* species complex (DUPONT et al., 2007) was reported. In addition, representatives of six fungal genera were found in the cave: *Chrysosporium, Gliocladium, Gliomastix, Paecilomyces, Trichoderma* and *Verticillium*. While no species identification was provided, the data reported by these authors suggest a strong correlation between cave fungi and arthropods because these fungal genera contain many entomopathogenic (insect infecting) species (SAMSON et al., 1988). Species of *Chrysosporium, Gliocladium, Paecilomyces,* and *Verticillium* have been isolated from larval and adult cadavers of cave crickets (GUNDE-CIMERMAN et al., 1998), and the association between fungi and insects in caves was recently reported by KUBÁTOVÁ AND DVORÁK (2005). These studies indicated the need for a detailed molecular study to decipher the origin of the microbial communities thriving in the cave.

3. Molecular Ecology

We collected eleven samples between April 2006 and January 2007 in different halls and galleries of Lascaux Cave. The Painted Gallery, Great Hall of Bulls, Chamber of Felines and Shaft of the Dead Man were selected to represent the different cave microenviroments. The samples included areas with white colonization, black stains, areas not apparently colonized (and therefore considered as references) and an area cleaned with biocides in 2004, without apparent colonization. Bacterial DNA was extracted from each sampling using a method adapted to small soil quantities as described previously (BASTIAN et al., 2009).

From the eleven samples, 696 clones were retrieved. Rarefaction analysis showed 90% clone coverage indicating that the majority of the bacterial diversity was detected. A considerable majority of the clone sequences retrieved could be assigned to defined taxa, from which the 10 most numerically dominant covered 533 clones or 76.6% of the total number of clones examined (Table 1) The two most abundant taxa were *Ralstonia* and *Pseudomonas*. The two most abundant phylotypes in the cave were *Ralstonia mannitolilytica* and *Ralstonia pickettii*, which together represent 17.8% of the total clones. It is noteworthy that only 5 sequences of *Pseudomonas fluorescens,* a species which was reported to be abundant in the cave (ORIAL AND MERTZ, 2006) were recorded. In contrast, 77 and 47 sequences corresponding respectively to *R. mannitolilytica* and *R. pickettii* were detected. There is unfortunately no information on the bacteria present in Lascaux Cave before benzalkonium chloride treatments and therefore a comparison of these results to the pre-tourism microbial communities cannot be performed. However, the data suggest that years of benzalkonium chloride treatments in Lascaux Cave may have selected a mixed population of *Ralstonia* and *Pseudomonas*, both highly resistant to the biocide.

Taxa	Total No. of clones (%)
Ralstonia	207 (29.7)
Pseudomonas	167 (24.0)
Escherichia	28 (4.0)
Achromobacter	25 (3.6)
Afipia	22 (3.2)
Ochrobactrum	20 (2.9)
Legionella	19 (2.7)
Alcaligenes	15 (2.2)
Stenotrophomonas	15 (2.2)

To gauge the fungal diversity in Lascaux Cave, we similarly produced and analyzed a clone library of fungal 18S rDNA sequences. Six hundred and seven clones were partially sequenced (750 bp) and sorted into phylotypes. The 10 most abundant phylotypes represented 59.2% of the clones (Table 2). Only two out of these ten phylotypes are soil fungi: *Tricholoma saponaceum* and *Kraurogymnocarpa trochleospora*, while the others are entomophilous fungi, including the well-known entomopathogens *Isaria farinosa*, *Engyodontium album*, *Geosmithia putterillii*, etc. This indicates that entomopathogenic fungi play an important role in the cave and arthropods contribute to the dispersion of spores.

Phylotypes	Total No. of clones (%)
Penicillium namyslowskii	79 (13.0)
Isaria farinosa	53 (8.7)
Aspergillus versicolor	37 (6.1)
Tolypocladium cylindrosporum	32 (5.3)
Tricholoma saponaceum	30 (4.9)
Geomyces pannorum	28 (4.6)
Geosmithia putterillii	28 (4.6)
Engyodontium album	28 (4.6)
Kraurogymnocarpa trochleospora	28 (4.6)
Clavicipitaceae sp.	17 (2.8)

4. Arthropod Ecology

In the last year, among other cavernicole arthropods, the springtail *Folsomia candida* (Collembola, Isotomidae) was found in Lascaux. This is a cosmopolitan opportunistic troglophile (facultative cavernicole, frequently completing its whole life cycle in caves, but not confined to this habitat) recorded from caves all over the world (PALACIOS-VARGAS, 2002). It is generally accepted that this springtail is found in cave sites which are not occupied by cave-adapted species and in disturbed and artificial areas. This fits well with the history of Lascaux as an example of ecologically disturbed site where *F. candida* likely was attracted from the top soil litter to the cave by the food source that represented the fungal outbreak.

Collembola are largely mycophagous and abundant *F. candida* specimens and were observed feeding on black stains (Fig. 2). *F. candida* prefer feeding on melanised fungal species (SCHEU AND SIMMERLING, 2004) over hyaline fungi, but they also feed on *Fusarium* hyphae (SABATINI AND INNOCENTI, 2000), several *Pseudomonas* spp., including *P. fluorescens* (THIMM et al., 1998) and nematodes (LEE AND WIDDEN, 1996).

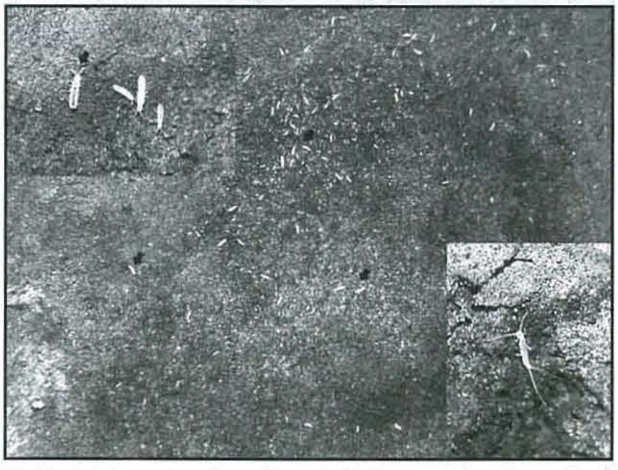

Table 2: Most abundant fungal phylotypes in Lascaux Cave.

Folsomia candida is a known vector for microorganisms. DROMPH (2003) reported that collembola can carry entomopathogenic fungi, and GREIF AND CURRAH (2007) isolated species of the fungal genera *Acremonium, Beauveria, Cladosporium, Cryptendoxyla, Geomyces, Gliocladium, Hormiactis, Leptographium, Oidiodendron, Penicillium,* and *Verticillium* from collembola. The high density of collembola that come in contact with bacterial cells, mycelial fragments and spores suggest that these arthropods can help disperse bacteria (SCHEU

AND SIMMERLING, 2004) and fungi (THIMM et al., 1998) throughout an environment. In addition, the gut of *F. candida* is a selective habitat and a vector for microorganisms (THIMM et al., 1998).

SABATINI et al. (2004) showed that some collembola preferred *Fusarium* as food and that a few colonies of *Fusarium* developed from their fecal pellets. We have observed that when *F. candida* is feeding on Lascaux black stains, and on peat debris in the laboratory, they have produced black fecal pellets. Also SAWAHATA (2006) has observed the production of black fecal pellets when collembolans consumed the hymenial area of agaric fruit bodies. Such fecal pellets can contain fungal spores that germinate once deposited on the wet rock surface. While the ability of spores to germinate may be reduced by gut passage, it is not uncommon for almost all fecal pellets produced by arthropods to contain some germinable spores (THIMM et al., 1998; WILLIAMS et al., 1998). Therefore, it could be possible that collembolans contribute to the sudden appearance of black stains everywhere in Lascaux Cave, although this hypothesis needs to be confirmed.

The presence of *F. candida* in Lascaux Cave after the first fungal outbreak may be explained by its feeding preferences. Fungi produce volatile compounds that are potentially attractive to collembola (BENGTSSON et al., 1988). Grazing on the black stains might be a consequence of the presence of melanised fungi in the black stains. Indeed these are also the most palatable fungi for mites (SCHNEIDER AND MARAUN, 2005) which suggest that a survey for this group of arthropods should be carried out in the cave. Interestingly, members of the family Campodeidae (Diplura) were observed in the black stains (Figure 2). Most Diplura are predators and their diet includes collembola and mites. They may also survive on vegetable debris and fungal mycelia.

5. Conclusions

This study raises intriguing questions about the past, present and future management of show caves. In fact, microorganisms in Lascaux Cave grow as biofilms which consist of assemblages containing many species of bacteria, fungi, protozoa, etc. Our research suggests that as a result of years of biocide treatments, the indigenous microbial communities of Lascaux Cave have been replaced by microbial populations selected for by biocide application.

It appears that arthropods contribute to the dispersion of fungal spores inside the cave, likely by contact with their bodies, dissemination of their fecal pellets and growth on their cadavers. The composition of the fungal community is largely influenced by arthropod colonization and activity, with the collembola of primary significance. Coleoptera likely play a role in fungal community structure, however, further studies are needed to verify this and the degree of association of *Geosmithia* species with cave beetles.

The black stains in Lascaux Cave seem to be related to the presence and grazing effects of the cavernicole population. A careful study of the arthropods, the fungal ecology and the dispersion patterns is needed in order to complete our understanding of the food web and to help confirm the suggested origin of black stains in the cave.

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