CULTURE-INDEPENDENT ANALYSES OF BACTERIAL COMMUNITIES ON PALEOLITHIC PAINTINGS AND SURROUNDING ROCK WALLS IN KARSTIC CAVES (ALTAMIRA, TITO BUSTILLO, LA GARMA AND LLONÍN)

Claudia Schabereiter-Gurtner,[1] Cesareo Saiz-Jimenez,[2] Guadalupe Piñar,[1] Werner Lubitz,[1] Sabine Rölleke[1,3]

[1]***Institut für Mikrobiologie und Genetik, Universität Wien, Austria.*** [2]***Instituto de Recursos Naturales y Agrobiologia, CSIC, Sevilla, Spain.*** [3]***Genalysis GmbH, Luckenwalde, Germany***

Paleolithic painting microbiology deserves attention, since it has been reported that microorganisms can affect painting pigments. Caves represent nutrient-poor ecosystems subjected to stable and low temperatures and although providing extreme environments for life, they are inhabited by a variety of microorganisms. However, few studies on cultivated bacteria in caves have been conducted so far. While these cultivation-based studies most probably revealed just a very minor and not representative part of cave population, culture-independent identification methods allowed the detection of unexpected and unknown bacteria and gave insight into a greater bacterial taxonomic diversity in caves (Engel et al. 2001; Holmes et al. 2001; Northup et al. 2000; Vlasceanu et al. 2000). Recently, the combination of PCR amplification of bacterial 16S rRNA genes (16S rDNA), phylogenetic sequence analyses, and genetic community fingerprinting by denaturing gradient gel electrophoresis (DGGE) was shown to be a proper method to study the bacterial communities on the valuable Paleolithic paintings and on the rock surfaces near the paintings in four Spanish caves of Altamira, La Garma, Llonín, and Tito Bustillo (Schabereiter-Gurtner et al. 2001, 2002a, b, c).

Altamira cave (Santillana del Mar, Cantabria, Spain) is known as the Sistine Chapel of Quaternary Art. The famous Polychromes Hall, discovered in 1879, is decorated with a herd of bisons surrounded by horses and deer (about 16,000 years old). Altamira cave has been visited extensively in the past and received up to 3,000 visitors per day in the 1970's. However, these visits resulted in severe deterioration. Nowadays the cave is closed for the public and visitors are derived to a cave reproduction. Llonín cave, near the village of Llonín (Asturias, northern Spain) was adapted for local cheese storage and maturation in 1957. Between 1987-1997 the cave was closed and only visited for archaeological excavations. In the lower part of the cave several primitive abstract figures in red and black colour dating back to the Magdalenian period are present (estimated age 22,000-10,000 BC). La Garma cave was discovered in 1995 under a hill near the village of Omoño (Cantabria, northern Spain) and is part of a complex karstic system of galleries situated at different heights and comunicating by vertical chasms. The cave represents an extensive paleolithic settlement with paintings from the initial Upper Paleolithic to the Magdalenian period. The valuable paintings show horses, bisons, hinds, non-figurative traces and hand prints. The cave is closed and only accessible for archaeological excavations. Tito Bustillo cave (Ribadesella, Spain) contains valuable Paleolithic paintings, which date back to 15,000-20,000 years. An extraordinary collection of paintings can be found on the Paintings Panel, situated in a hall near the end of the cave. The panel presents paintings showing horses, reindeers, red deer stags, tectiforms, hinds and bovines. Since 1969 the cave is open for the public.

In all four caves, rock wall surfaces, speleothems, soils and even parts of the Paleolithic paintings are covered by conspicuous bacterial growth in the form of round colonies of different colours (about 1-2 mm diameter) and patches. For microbiological analyses, 14 samples representing macroscopical colonies and material without apparent bacterial colonization taken from the rock walls and paintings were taken. Genomic DNA was extracted without prior cultivation and the bacterial communities were analyzed by PCR, ribosomal clone libraries and DGGE

community fingerprinting (Schabereiter-Gurtner et al. 2001). Figure 1 shows the DGGE-fingerprints of the bacterial communities. For phylogenetic analyses of individual bacterial members, 147 clones were sequenced and compared with known 16S rDNA sequences listed in the EMBL database. Microorganisms were phylogenetically most closely related to cultured bacteria (34%) and to environmental 16S rDNA clones (66%), showing the broad spectrum of unknown and yet to be cultivated bacteria in caves. The distribution of the phyla in the different caves is shown in Table 1.

Table 1. Distribution of clones within the major phylogenetic groups of samples taken in four Spanish caves containing valuable Paleolithic paintings

Number of 16S rDNA clones and their closest relation to the major phylogenetic groups	Altamira	Llonín	La Garma	Tito Bustillo
Proteobacteria	11	18	20	20
Green non-sulfur bacteria	1	-	1	3
***Planctomycetes* order**	1	-	-	1
***Cytophaga/Flexibacter /Bacteroides* division**	2	2	2	1
***Acidobacterium* division**	5	-	16	12
Low G+C Gram-positive bacteria		1	8	-
Actinobacteria	1	6	11	4

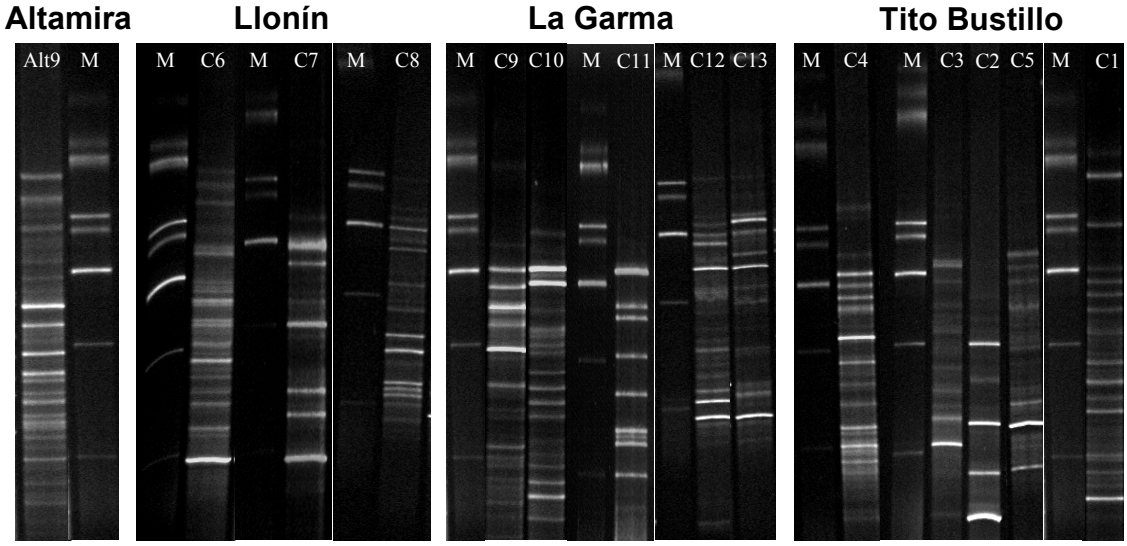

Fig. 1. Detail of the ethidium-bromide-stained 16S rDNA DGGE community fingerprints of samples taken in four Spanish caves. DGGE allows the separation of a mixture of partial 16S rDNA amplicons according to their sequences in polyacrylamide gels containing a gradient of denaturants. Each DGGE band represents a bacterial taxon.

Gram-negative bacteria were the most abundantly identified bacteria, from which *Proteobacteria* dominated in all samples (47%), followed by members of the *Acidobacterium* division (22.4%). Bacteria affiliated with the *Proteobacteria* were most closely related to cultured and uncultured members of the *Chromatiaceae, Comamonadaceae, Ectothiorhodospiraceae, Geobacteraceae, Moraxellaceae, Pseudomonadaceae, Rhizobiaceae, Rhodospirillaceae, Sphingomonas* group, *Rhodocyclus* group, *Xanthomonas* group and to ammonia-oxidizers of the beta- and gamma-*Proteobacteria*. Some thereof were putatively chemolithoautotrophic proteobacteria related to sulfur-oxidizers, to putatively iron-oxidizing bacteria, ammonia-oxidizers, and to N_2-fixing chemoorganotrophic bacteria. Bacteria affiliated with the *Acidobacterium* division were uncultured bacteria from different soils. The *Acidobacterium* division is a recently described monophyletic phylum with only three so far cultivated species. In Llonín cave, no members of the *Acidobacterium* division were identified. The identification of members of the deep-branching *Acidobacterium* division in three of the four caves represents the first reports for subterranean environments. The remaining bacteria were related to the *Cytophaga/Flexibacter/Bacteroides* phylum (4.8%), green non-sulfur bacteria (3.4%), *Planctomycetales* (1.4%), low G+C Gram-positive bacteria (*Bacillus/Clostridium* group) (6.1%), and *Actinobacteria (Actinosynnemataceae, Pseudonocardiaceae, Micrococcaceae, Nocardioidaceae,* and *Streptomycetaceae*) (15%). The abundance of Gram-positive bacteria was significantly low in all four caves.

The strong bacterial growth on the valuable paintings is astonishing and alarming —bacterial communities on wall paintings have biocorrosive potential and may cause irreversible damages. However, in general the culture-independent approach allows only vague statements about the physiological properties of identified bacteria and the ecological and biodeteriorative role they might play on Paleolithic paintings.

Acknowledgements

This research was supported by the European Comission, contract ENV4-CT95-0104 and the Austrian-Spanish Cooperation Agreement HU 1997-0035. The facilities provided by the archaeologists responsible of the caves sampled are acknowledged.

References

Engel A.S., Porter M.I., Kinkle B.K., Kane T.C. (2001). Ecological assessment and geological significance of microbial communities from Cesspool Cave, Virginia. Geomicrobiol. J. 18: 259-274.

Holmes A.J., Tujula N.A., Holley M., Contos A., James J.M., Rogers P., Gillings M.R. (2001). Phylogenetic structure of unusual aquatic microbial formations in Nullarbor caves, Australia. Environ. Microbiol. 3: 256-264.

Northup D.E., Dahm C.N., Melim L.A., Spilde M.N., Crossey L.J., Lavoie K.H., Mallory L.M., Boston P.J., Cunningham K.I., Barns, S.M. (2000). Evidence for geomicrobiological interactions in Guadalupe caves. J. Caves Karst. Stud. 62: 80-90.

Schabereiter-Gurtner C., Piñar G., Lubitz W., Rölleke S. (2001). An advanced molecular strategy to identify bacterial communities on art objects. J. Microbiol. Meth. 45: 77-87.

Schabereiter-Gurtner C., Saiz-Jimenez C., Piñar G., Lubitz W., Rölleke S. (2002a). Cave Paleolithic paintings harbour complex and partly unknown microbial communities. FEMS Microbiol. Lett. 211: 7-11.

Schabereiter-Gurtner C., Saiz-Jimenez C., Piñar G., Lubitz W., Rölleke S. (2002b). Phylogenetic 16S rRNA analysis of Tito Bustillo Cave, Spain, and its Paleolithic paintings reveals the presence of complex and partly unknown microbial communities. Environ. Microbiol. (In press).

Schabereiter-Gurtner C., Saiz-Jimenez C., Piñar G., Lubitz W., Rölleke S. (2002c). Phylogenetic diversity of bacteria associated with Paleolithic paintings and surrounding rock walls in two Spanish caves (Llonín and La Garma). Submitted to Microb. Ecol.

Vlasceanu L., Sarbu S.M., Engel A.S., Kinkle B.K. (2000). Acidic cave-wall biofilms located in the Frasassi Gorge, Italy. Geomicrobiol. J. 17: 125-140.

Forthcoming Activities

COALITION Workshop: Sustainable Conservation: An European Concern

This workshop, the last of the Concerted Action, will be held in Florence, Italy, on November the 7th, 2002. This workshop will consist in a round table held by the following participants:

May Cassar (CSH, London)
Johanna Leissner (EC, Brussels)
Cristina Acidini (OPD, Florence)
Mauro Matteini (ICVPC, Florence)
Antonio Paolucci (Polo Museale Fiorentino, Florence)
Domenico Valentino (BAPPSAD, Florence)
Daniela Pinna (LSSPSA, Bologna)
Umberto Baldini (UIA, Florence)
Rocco Mazzeo (Università di Bologna)
Roberto Franchi (Università di Urbino)
Paolo Bianchini (ST Opera del Duomo, Florence)
Roberto Balzarotti (Phase, Bologna)
Lucia Nucci (ARI)

COALITION Advanced Course on Biodeterioration of the Cultural Heritage

During the days 8 and 9 of next November an Advanced Course on Biodeterioration of the Cultural Heritage will be held in Florence, Italy, with the following programme:

8 November 2002

8.30 Presentation - C. Saiz-Jimenez

SESSION 1 - BIODETERIORATION PROCESSES
9.00-10.00. On organic substrates - N. Valentin
10.00-11.00.On inorganic substrates - L. Tomaselli

SESSION 2 – MODERN DIAGNOSTIC TECHNIQUES
11.30-12.30 On isolates - J. Swings
12.30-13.30 Community analysis - S.. Roelleke

SESSION 3 - INTERVENTION PROCEDURES
14.30-15.30 Prevention - D. Pinna
15.30-16.30 Treatments - O. Salvadori

SESSION 4 – BIOREMEDIATION
17.00-18.00 Cleaning - C.Sorlini
18.00-19.00 Consolidating - P. Tiano

9 November 2002

SESSION 5 - BIOHAZARD IN RESTORATION
9.00-10.00 Air monitoring - M.P. Nugari
10.00-11.00 Allergenic, pathogenic and Toxicity M. Salkinoja Salonen

SESSION 6 - – COALITION QUESTIONNAIRE
11.30-12.30 Illustration of the results - C. Kyi