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Experiment Report Form

The double page inside this form is to be filled in by all users or groups of users who have had access to beam time for measurements at the ESRF.

Once completed, the report should be submitted electronically to the User Office using the **Electronic Report Submission Application:**

http://193.49.43.2:8080/smis/servlet/UserUtils?start

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Reports can now be submitted independently of new proposals – it is necessary simply to indicate the number of the report(s) supporting a new proposal on the proposal form.

The Review Committees reserve the right to reject new proposals from groups who have not reported on the use of beam time allocated previously.

Reports on experiments relating to long term projects

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Should you wish to make more general comments on the experiment, please note them on the User Evaluation Form, and send both the Report and the Evaluation Form to the User Office.

Deadlines for submission of Experimental Reports

- 1st March for experiments carried out up until June of the previous year;
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Instructions for preparing your Report

- fill in a separate form for each project or series of measurements.
- type your report, in English.
- include the reference number of the proposal to which the report refers.
- make sure that the text, tables and figures fit into the space available.
- if your work is published or is in press, you may prefer to paste in the abstract, and add full reference details. If the abstract is in a language other than English, please include an English translation.

ESRF	**Experiment title:** STUDY OF BIOMINERALIZATION OF CALCITE AND APATITE BY BACTERIA EXTRACTED IN PREHISTORIC CAVES – THE CASE OF THE GRANDE GROTTE D'ARCY-SUR-CURE	**Experiment number**: EC161
Beamline: ID21	**Date of experiment**: from: 09/03/2007 to: 13/03/2007	**Date of report**: 01/08/2007
Shifts: 12	**Local contact(s)**: Emilie Chalmin	*Received at ESRF:*

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Report:

A multi-disciplinary project was started to understand the formation of different calcium carbonate types in the decorated cave of Arcy-sur-Cure in order to evaluate its role on prehistoric paintings. In previous work, about forty natural samples at different locations in the cave were studied using mainly laboratory-based analytical methods and classical microbial as well as biomolecular analyses to characterise the chemical composition, structure and an eventually biological origin of these samples [1]. Different microstructures could be evidenced in the calcites sampled in the cave. These features are induced by varying growth mechanisms that can be completely abiotic but also show biotic contributions. Indeed, bacteria could be isolated from the natural calcite samples indicating an important microbiological activity. The diversity of the bacteria was already evidenced thanks to effective microbiological method. However, their possible role in the calcite growth mechanisms needs to be elucidated unambiguously. In addition, calcite interaction with compounds of iron oxide bearing paint layers of the prehistoric rock art was evaluated. However, on the basis of the natural calcites available in the cave it could not be investigated if other pigments used for rock art show the same behaviour [2].

During this beamtime, synthetic calcites prepared using varying abiotic and biotic conditions were studied. They were synthesised in solution in biotic or abiotic condition with various cations or with varying CO_2 pressure. It is expected that the comparison of the characteristics of natural calcites with those of synthetic ones (especially those prepared in biotic media) gives additional insights into the calcite growth mechanisms and especially into the role of microorganisms in this phenomenon.

Synthetic and natural samples were analysed by means of micro-XRF at 4.15 keV (Ca K-edge). The microbeam was focused thanks to a zone plate to 0.34 x 0.75 μm^2. The first objective was to map Ca and P distribution in the biotic synthetic samples with a good spatial resolution. And the second aim was to acquire Ca K-edge XANES spectra on the series of synthetic and natural samples in order to confirm the mineral phase, to detect structural information and to discriminate phosphate and carbonate phases in biotic samples.

Ca K-edge micro-XANES spectra were collected under high resolution conditions in energy (~ 0.5 eV) between 4.00 and 4.15 keV. The XANES spectra were acquired in fluorescence mode thanks to a SDD and in transmission with a photodiode.

Results on natural samples from the "Grande Grotte"of Arcy-sur-Cure

Micro-XANES on different layers of the stalactite section NC#05 shows that similar spectrum features are observed for white opaque layers at the inside and at the outside of the section with respect to the paint layer. Their shape can be clearly distinguished from the XANES feature of the translucent calcite layers inside and outside compared to the paint layer (Fig.2b). A variation in intensity is observed in the fine structure of the white line (4.051 – 4.055 keV) of the XANES feature. Even in the clay- and pigment-bearing layers visible on the micrograph (Fig.2a), the contribution of calcite can be observed. It is possible to distinguish the calcite associated with the clay, similar to the translucent one and the calcite associated with the pigment, similar to the opaque one. This finding confirms the presence of two layers trapped in the calcite layers of NC#05: one containing mainly iron oxide and another that principally bears clay. However, it seems that XANES intensity variations (particularly for the white line) observed on opaque and translucent layers of NC#05 are not found on the corresponding powdered samples. Information on crystal orientation is lost and crystal size might be changed by powder sampling and preparation. Therefore, effects of crystal sizes and orientation that seems to be reflected in the XANES structure of thin sections are lost when analysing powders.

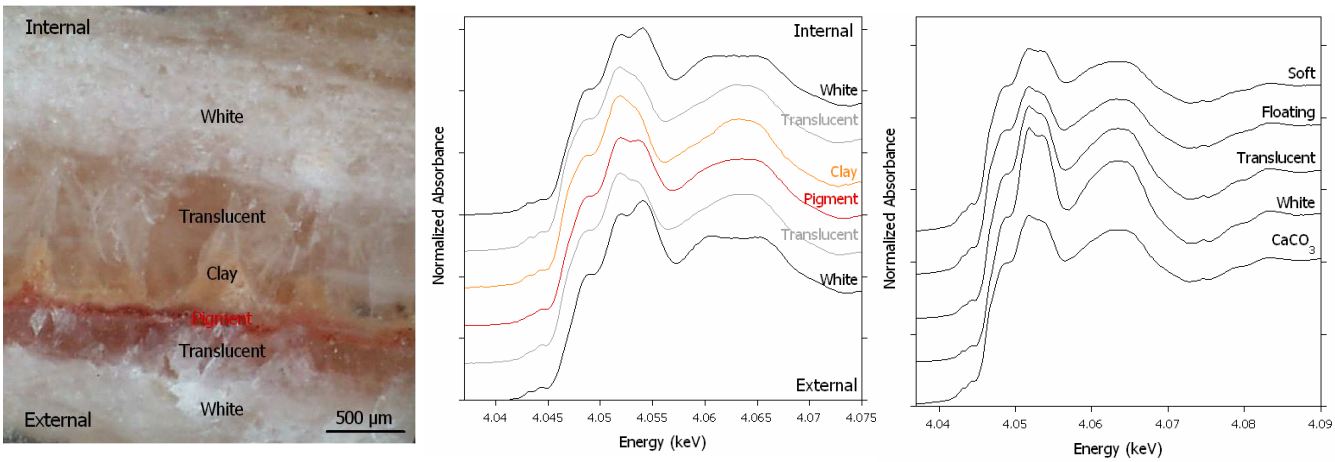

Figure 2. a) Cross section of the stalactite (NC#05) containing a prehistoric painting layer observed by binocular. b) Ca K-edge XANES spectra acquired on the cross section in fluorescence mode. c) Ca K-edge XANES spectra acquired on powder samples coming from the cave.

Results on biotic samples

About twenty biotic calcites (BC#1-20) were synthesized using the bacteria *Bacillus sp.* (B50) and P*seudomonas fluorescens* (B51), isolated from natural calcites taken from the cave and then cultivated in two media (Nutrical N and modified Nutrical N'). For each medium, solutions were sampled at time zero (t_0) without bacteria, t_0 with bacteria, after 2, 10, 22 and 30 days of incubation at 22°C. The entire samples (biomass and organic and mineral residual) were centrifuged, rinsed with distilled water and then dried. A part of the samples was heated at 500°C during 3h to eliminate the organic residuals. A first XRD analysis evidenced that calcite and another mineral phase, hydroxyapatite, a calcium phosphate, is systematically formed in all biotic samples next to calcite.

Forteen of these biotic samples were observed either in powder, in pellet or after an microtome preparation (20 µm of thickness) to get transmission data. For each sample, the systematic presence of P associated with Ca was confirmed by mapping (see Fig 3a) and the presence of a mixture of phosphate and carbonate phases was evidenced (Fig.3b). No presence of pure calcite grain was detected, but micro-XANES spectrum presents a mixture of calcite and HAP. With a peak at 4.060 keV, the XANES shape is near the calcite structure and the presence of a pre-edge without doublet is more specific of the HAP structure (Fig.3b). For example, micro-XANES features measured on specific points on the map (Fig.3a) indicate a

mixture calcite-HAP confirmed by deconvolution calculation (Fig.3b). The quantity of calcite is estimated around 50 % for two points and a major phase of HAP (99%) is detected in another point, consistent with the high P concentration. For the bigger grain observed in this sample (point 1) the calcite/HAP ratio (24.6 % calcite) seems to be underestimated. Indeed, the recalculated spectrum (Fig.3c) doesn't fit well with the experimental data. This difference seems to indicate the presence of another compound, not considered in this calculation. It is necessary to test contributions of other phosphates, calcium carbonate or mixed phosphate-carbonates references to improve the agreement of experimental and calculated data. XRD analysis is necessary to determine the structural information about this compound.

XANES spectra realized on the both bacteria show the absence of calcite and the presence of a pure calcium phosphate (Ca_3PO_4), which could provide from the phospholipids in the bacteria, the main constituents of the bacteria membrane. The complementary analysis by micro-XRD on ID18F could provide more information about the crystallinity of this component.

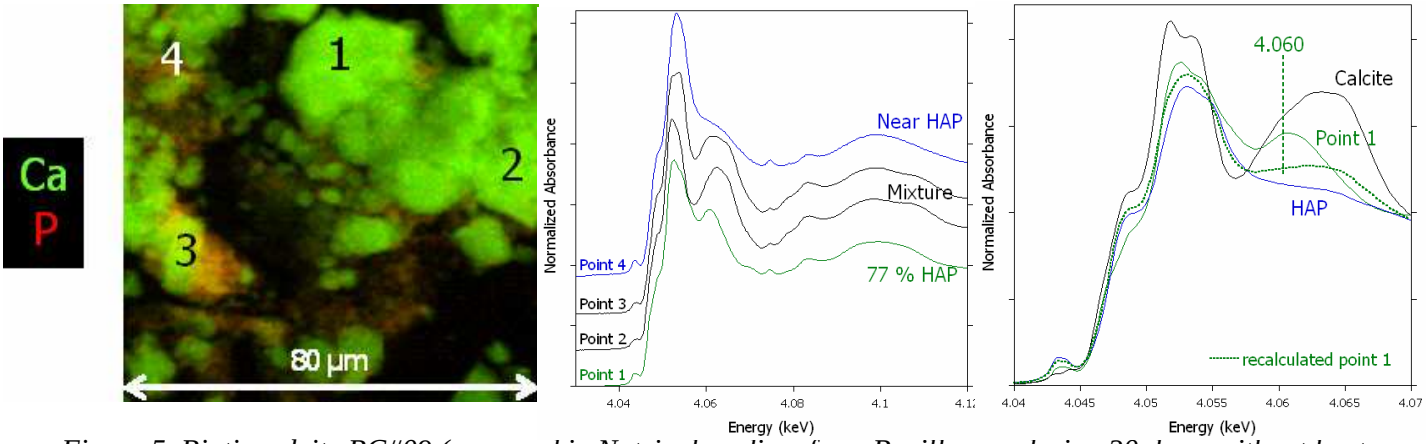

Figure 5. Biotic calcite BC#09 (prepared in Nutrical medium from Bacillus sp. during 30 days, without heat-treatment): a) Mapping at 4.1 keV (pixel size: 0.5 x 0.5 µm^2) and location of various points for XANES , b) Ca K-edge micro-XANES spectra on different points of the mapping (in transmission mode, beam size: 0.34 x 0.75 µm^2), c) Comparison between reference Ca K-edge XANES of calcite, hydroxyapatite (HAP) and micro–XANES on the point 1 and recalculated spectrum from linear deconvolution calculation (in transmission mode).

To conclude, these investigations of biotic calcites evidenced the calcifying properties of the bacteria *Bacillus sp*. and *Pseudomonas fluorescens* isolated from natural calcite samples originating from the "Grande Grotte" of Arcy-sur-Cure. However, the culture conditions used did not exactly correspond to that of the cave. Therefore, conclusions on the role of bacteria in the growth of natural calcite are difficult to draw. The origin of the hydroxyapatite phase and its role in the formation of calcite has still to be clarified. The understanding of the exact formation mechanism of the biotic calcite and apatite would be of great importance because it would allow defining a marker in biologically formed calcites.

A part of these results was presented during the international conferences Technart 2007 at Lisboa (Portugal) in April (25-27) and in a publication submitted to the proceeding of this conference that will be published in X-ray Spectrometry [3]. In addition, the results will be presented at the French conference "Sciences des Matériaux du Patrimoine Culturel" in December 2007 in Paris organized by the French Ministry of Culture on the financed PNRC projects. Together with the conference, there will be a publication in TECHNE.

[1] Chalmin E, D'Orlyé F, Zinger L, Charlet L, Geremia R, Orial G, Menu M, Baffier D, and Reiche I, *J Geol Soc London*. 2007; **279**: p. 185-197.
[2] Reiche I, Chalmin E, d'Orlyé F, Sansot E, Menu M, Charlet L, Orial G, Geremia R, Baffier D, and Girard M. in *36th International Symposium on Archaeometry, ISA2006*. 2006. Quebec city: Laval's series of Publications in Archeometry.
[3] Chalmin E, Sansot E, Orial G, Bousta F, Reiche I. Combination of Microanalysis and synthesis for the Understanding of calcite formation on prehistoric paintings: the case of the Grande Grotte, Arcy-sur-Cure (Yonne, France). submitted to *X-ray Spectrometry*