Study of Interactions Between Microbes and Minerals by Scanning Transmission X-Ray Microscopy (STXM)

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Abstract. Scanning Transmission X-ray Microscopy (STXM) and Transmission Electron Microscopy (TEM) were combined to characterize various samples of geomicrobiological interest down to the nanometer scale. An approach based on energy-filtered imaging was used to examine microbe-mineral interactions and the resulting biominerals, as well as biosignatures in simplified laboratory samples. This approach was then applied to natural samples, including natural biofilms entombed in calcium carbonate precipitates and bioweathered silicates and facilitated location of bacterial cells and provided unique insights about their biogeochemical interactions with minerals at the 30-40 nm scale.

INTRODUCTION

Prokaryotes, which include Bacteria and Archeae, are found almost everywhere on Earth's surface, including ecosystems with extreme temperatures (from -160°C to 121°C), pH's, and salinities. They have been shown to catalyze a large variety of chemical reactions involving almost all the elements from the periodic table. In addition, there is a general belief that they have been populated Earth for more than 3 billion years, which in addition to their relatively short generation time, implies that they have achieved a huge accumulated biomass. These facts suggest that Life may have played an important role in the evolution of Earth's surface chemistry. However, as most of the geochemical processes occurring on Earth can *a priori* (i.e. based on basic thermodynamic considerations) be explained by either abiotic or biotic mechanisms, the real impact of microbes on those processes has yet to be assessed. To do so requires investigations of both organic compounds and minerals at the scale at which microbes work, i.e. the submicrometer scale, which is a major methodological challenge. Here, we illustrate through selected geomicrobiological studies, a general approach combining TEM and STXM that provides useful information on both minerals and organic compounds associated with microbial organisms at the 30-nm scale. We address first the issue of locating microbes in complex (heterogeneous and diluted) natural samples and then use spectromicroscopy methods to characterize geomicrobiological reaction products.

EXPERIMENTAL

STXM observations were performed at ALS branch line 11.0.2.2 following the same procedures as previously described [1]. The synchrotron storage ring was operated at 1.9 GeV and 200-400 mA stored current. A 150 l/mm grating and 20 μm exit slit were used for carbon and nitrogen K-edge imaging and spectroscopy, providing a theoretical energy resolution of 100 meV. Energy calibration was accomplished using the well-resolved 3p Rydberg peak at 294.96 eV of gaseous CO_2 for the C K-edge. Calibration at the N K-edge was made by using the N $1s \rightarrow \pi*$ (401.1 eV) transition of atmospheric N_2.

TEM observations were performed on a Jeol 2010F microscope operating at 200 kV and equipped with a field emission gun, a high resolution UHR pole piece, and a Gatan energy filter GIF 200. EELS analyses were performed following the same procedures as described previously [2]. The energy resolution was about 1.3 eV as measured by the full width at half

maximum of the zero-loss peak. Spectra were corrected from plural scattering using the Egerton procedure available with the EL/P program (Gatan).

RESULTS & DISCUSSION

Locating Microbes in Rocks

NEXAFS spectra of diverse cultured bacteria including the Gram negative bacteria *Caulobacter crescentus* (α-Proteobacteria), *Shewanella oneidensis* (γ-Proteobacteria)*,* and *Synechococcus leopoliensis* (Cyanobacteria), and the Gram-positive bacterium *Bacillus subtilis* were measured at the C K-edge. They all display very similar features (e.g., peaks at 285.2 eV, 286.8 eV, 288.2, 289.5 eV) resulting from the absorption of x-rays by the various biochemical compounds that compose cells. Absorption by proteins at 288.2 eV produces the dominant spectral feature (Fig. 1). The similarity of NEXAFS spectra is not surprising given the shared basic chemistry of living cells, all of which contain proteins, polysaccharides, and nucleic acids. We suggest that other microbial
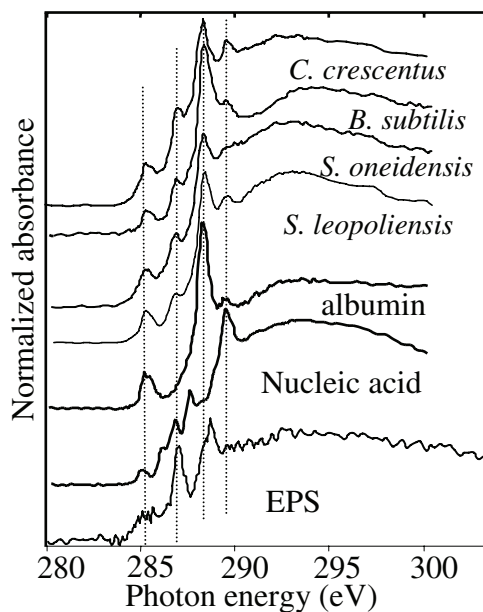
FIGURE 1. Carbon K-edge NEXAFS spectra of *Caulobacter crescentus, Bacillus subtilis, Shewanella oneidensis,* and *Synechococcus leopoliensis*. Reference spectra of nucleic acid (from [17]), albumin (taken as a model for protein, [17]), and EPS (mostly polysaccharides) are shown for comparison. Dashed lines denote peaks at 285.2, 286.5, 288.2, 289.5 respectively. (from [1]).

groups that have not yet been investigated will have C K-edge spectra similar to those of the bacteria considered here. N K-edge NEXAFS spectra were also measured on *C. crescentus* cells, showing peaks at 399 eV, 399.9 eV, and 401.2 eV (data not shown) and may provide an additional spectroscopic signature for microbial cells. However more work is required to understand the meaning of the different peaks in these spectra [3].

Whether NEXAFS spectra at the C and N K-edges are unambiguously characteristic of cells or not is an important issue that will require many more systematic analyses of natural samples. However, NEXAFS spectroscopy at the C K-edge has been extensively used for this purpose in the past, and many reference spectra exist in databases, including spectra of other abundant organic compounds in natural environments like humic acids (e.g., [4,5]). Based on this knowledge base, we believe that finding a micrometer-sized particle in natural samples with C K-edge and N K-edge NEXAFS spectra similar to those of microbes previously examined is strongly indicative that bacteria are present.

Using a protocol designed by Benzerara et al. [6], we obtained *C. crescentus* and *Ramlibacter tataouinensis* (β-Proteobacteria) cells fossilized experimentally by hydroxyapatite $[Ca_{10}(PO_4)_6(OH)_2]$ [1]. In the case of *R. tataouinensis*, the cells were aged for 4 years. NEXAFS spectra at the C K-edge and N K-edge of fossilized and aged cells are qualitatively similar to those measured on fresh cultures with the exception of an additional peak at 290.3 eV in C K-edge spectra of the fossilized and aged cells, which is characteristic of carbonate functional groups present in carbonate hydroxyapatite (Fig. 2).

Based on these observations, we tested the purported microbiological origin of several human and bovine arterial calcifications in which self-replicating, calcifying entities were previously detected and isolated and were referred to as nanobacteria [7]. There is a controversy concerning whether the entities < 200 nm in length are biomineralized microbes or minerals that nucleated on something other than a microbe (e.g., proteins or arterial cell membranes). NEXAFS spectra at the C K-edge, N K-edge, and Ca $L_{2,3}$-edge on calcified nanoparticles isolated from human and bovine samples revealed organics, likely peptides or proteins, specifically associated with hydroxyapatite minerals but failed to detect any other biochemical compound present in reference bacteria.

In another STXM-TEM study involving carbonate microbialites, we used the major absorption of proteins at 288.2 eV and energy-filtered imaging to map proteins in microbialites from Lake Van, Turkey [8]; such microbialites are highly mineralized carbonate sediments associated with a relatively high diversity of Bacteria [9]. Bright spots were observed on protein maps (Fig. 3) with filamentous or spherical morphologies and sizes in the sub-micrometer to micrometer size range compatible with those of

individual microbial cells or remnants of microbial cells. C and N K-edge NEXAFS spectra obtained on these spots were similar to those observed in the reference bacteria in similar intensity ratios. Based on both morphological and spectroscopic considerations, we argue that the peptide-bond map of the Lake Van microbialite samples shows the locations of living or fossilized microbial cells [8]. A similar approach was used on various samples containing bacteria and systematically allowed the location of cells.

Finding Traces of Life in Ancient Rocks

A significant effort has been made over the last 30 years to find traces of life in ancient rocks (> 2 to 3 billions years old) and to understand which metabolic pathways existed then. The presence of some microbial fossils was proposed based on carbon isotope geochemistry, optical microscopy, and/or Raman spectromicroscopy (e.g., 3.5B.Y. cyanobacteria fossils [10]), but their biological origin has been highly debated until very recently (e.g., [11]). Among the major difficulties in finding biosignatures in old rocks, is the difficulty of recognizing submicrometer-sized organic carbon particles in a highly mineralized, fine-grained matrix. A second difficulty is the partial transformation of carbon (e.g., graphitization) in rocks that have usually undergone temperature and pressure increases (i.e. metamorphism), which is commonly believed to erase indicators of biogenicity [12].

We have addressed the second issue by studying a Triassic (~225 Myr old) highly metamorphosed carbonate rock (340°C, 12 kbars, i.e. ~35 km of burial) during the formation of the Alps. Despite a relatively young geological age, this sample is particularly interesting as the metamorphic conditions it experienced are very well defined. STXM combined with TEM observations have shown the persistence of carbonaceous materials in these rocks associated with chemical, textural, and mineralogical heterogeneities that we interpreted as the products of primary biogenic nanostructures [13]. These observations suggest that remnants of biological structures can be preserved even in metamorphosed samples. Hence, it seems feasible to search for preserved organic carbon in old rocks, using the energy-filtered imaging capabilities of STXM in a way similar to that presented above, to characterize their carbon and nitrogen functional groups. Such biosignatures can be used to identify candidates for potential microfossils that can be then studied using other analytical techniques.

## What is the Impact of Microbes on Geochemical Transformations

Locating microbes in a sample is a necessary first step in geomicrobiological investigations of natural samples, but detailed characterization of the geochemical transformations induced by microbes is the major goal in such studies. The wide range of energy (i.e. 75-2150 eV) accessible with the 11.0.2 beamline at the ALS allows mapping the speciation of diverse chemical elements in microenvironments associated with microbes once they are located. We present two examples of this approach.

The role of microbes in the formation of carbonate deposits has been much debated in the past. We studied carbonate deposits from the alkaline Lake Van (Turkey), which is highly supersaturated with respect to aragonite. While a purely abiotic mechanism could be inferred from thermodynamic considerations, observations of a huge microbial biomass and diversity associated with these deposits suggest that life played a role in their formation. As previously shown (Fig. 3), most of the carbonates are not directly linked spatially with microbes. However, using C K-edge spectroscopy, we showed that microbially produced polysaccharides are pervasive in the carbonate deposits. Combining STXM observations with TEM and EELS measurements at the nm-scale, we proposed that polysaccharides are associated with the aragonite crystals at the nm-scale and that they may be responsible for the unusual morphology and texture (i.e. clustered, 30-100 nm-sized smooth globules) of these crystals in the Lake Van microbialites [8].

STXM can also provide information on the impact of microbes on metal redox speciation using the $L_{2,3}$ edges of transition metals (e.g., Mn, Fe, Cr) or metalloids (e.g., As). In particular, Fe $L_{2,3}$ edges have been extensively studied (e.g., [15]). After having located a microorganism on the surface of a meteoritic orthopyroxene, we measured the Fe L_3-edge NEXAFS spectra in a microorganism/Ca-carbonate/Fe^{2+}-silicate microcosm at a spatial resolution of less than 40 nm (Fig. 4) [16]. Iron spectra taken on the Fe^{2+}-silicate (area 1) showed a major peak at 707.8 eV, indicative of Fe^{2+} (Fig. 4). Iron-rich particles in the calcite cluster (area 2) displayed a major peak at 709.5 eV, which indicates that iron was oxidized after its release by dissolution of the silicate (Fig. 4). In the microorganism (area 3), however, the Fe L_3-edge shows a mixed iron valence (Fe^{2+} and Fe^{3+}) (Fig. 4). Several mechanisms can be proposed at this stage to explain this iron redox behavior, but whatever mechanisms are involved, the microorganism heavily impacts iron oxidation dynamics, resulting in a major modification of the silicate reactivity compared with a purely abiotic environment.
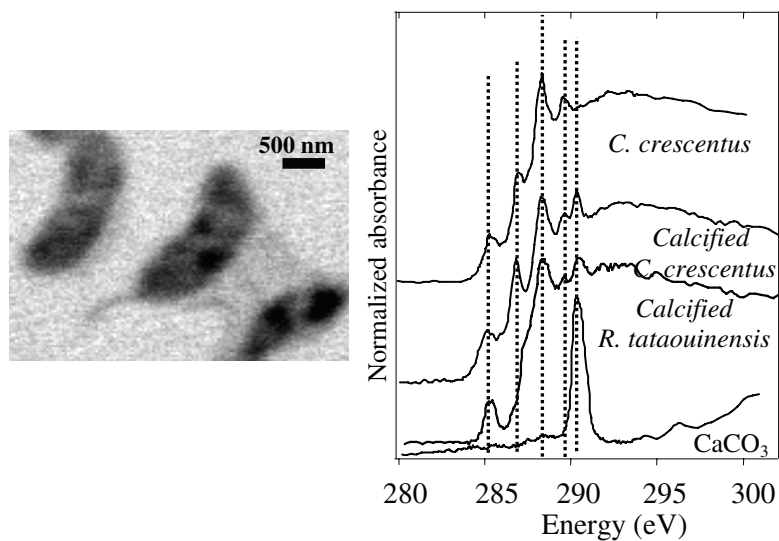
FIGURE 2. Spectromicroscopy on calcified *C. crescentus* and *R. tataouinensis* cells at the carbon K-edge. (a) STXM image taken at 288.2 eV, i.e. the resonance energy of amide groups in proteins. An appendage is observed on the cell in the middle of the picture and was also observed for many other cells. (b) NEXAFS spectra of non-calcified *C. crescentus* and calcified *C. crescentus* and *R. tataouinensis* cells. A reference spectrum of carbonates is shown for comparison. Dashed lines denote peaks at 285.2, 286.5, 288.2, 289.5, and 290.3 eV, respectively. (from [1])
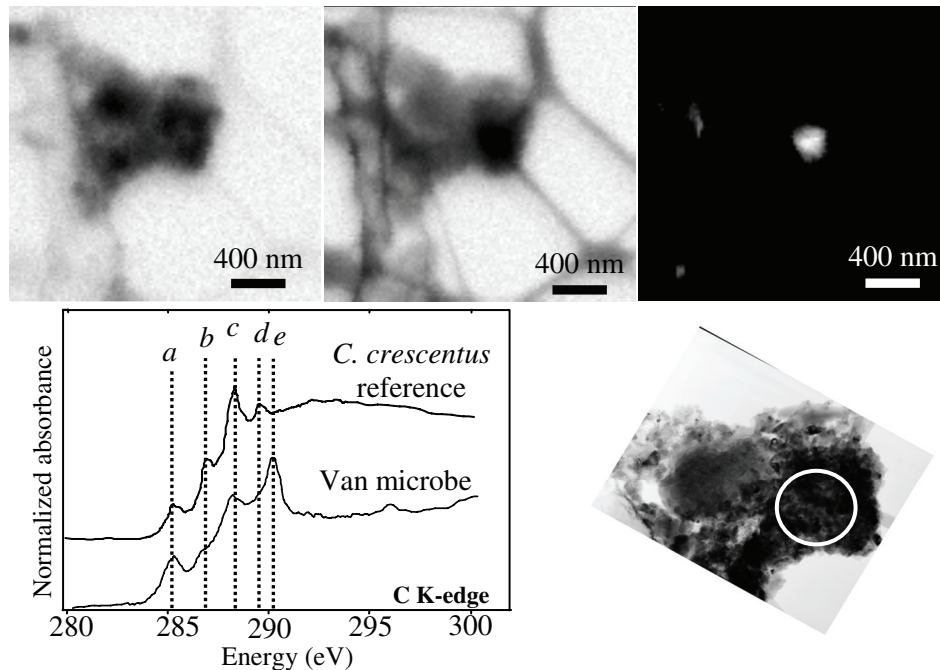
FIGURE 3. Locating microbes in Lake Van, Turkey carbonates. (upper left to right) STXM image taken at 280 eV, i.e. below the C K-edge; STXM image taken at 288.2 eV, i.e. the resonance energy of amide groups; Protein map; (lower left to right) NEXAFS spectra from the bright spot on the protein map. The NEXAFS spectrum of a reference bacterium is shown for comparison. Dashed lines denote peaks at 285.2, 286.5, 288.2, 289.5 and 290.3 eV, respectively; TEM image of the same area; the microbe is almost invisible due to its poor contrast compared with that of crystals covering the microbe. (from [8])
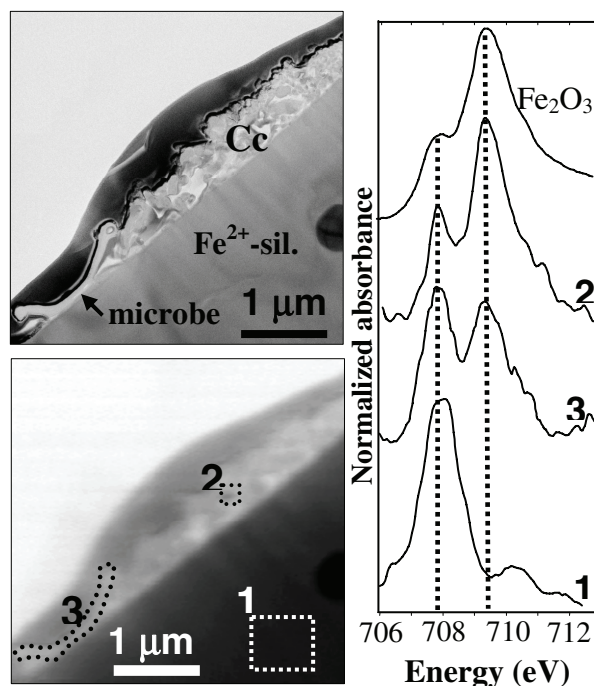
FIGURE 4. (Upper left) TEM image of a cross-section showing a microorganism (arrow), a calcite crystal cluster (Cc), and a Fe^{2+}-silicate. (Bottom left) Equivalent STXM image at 707.8 eV. (Right) Iron L_3-edge NEXAFS spectra from the silicate (area 1), the calcite cluster (area 2), the microorganism (area 3), and reference hematite, representing the Fe^{3+} endmember. Dashed lines represent the positions of Fe L_3 maxima for Fe^{2+} and Fe^{3+} at 707.8 and 709.5 eV, respectively. (from [16])

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