EUROPEAN SYNCHROTRON RADIATION FACILITY
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Experiment Report Form

	Experiment title: Subcellular imaging of Hg to decipher Hg methylation in bacteria	**Experiment number**: EV-157
Beamline: ID16B	**Date of experiment**: from: 30 September 2015 to: 5 October 2015	**Date of report**: 17 October 2015
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Report:

Scientific background and objectives:

Methylmercury (MeHg), mainly produced by sulphate-reducing bacteria (SRB), is biomagnified in aquatic food chains and poses a risk to human health. Very little is known regarding the mechanism of inorganic Hg (Hg(II) or IHg) uptake and methylation by these organisms as well as the demethylation process, in part because of the difficulty in measuring the intracellular Hg concentration and the location where the transformation takes place. Our objective was to image Hg in SRB bacteria using nano-X-ray fluorescence to clarify where the methylation and demethylation mechanisms occur. Some specific tasks were exciting and challenging since we were interested in collecting elemental images in fluorescende mode in cryogenic conditions and to collect some Hg L_3-edge XANES spectra in this mode to get insights on Hg chemical environment in the bacteria cell. These set-ups are being developped on the new ID16B beamline.

Experimental:

Two anaerobic strains were studied: strain *BerOc1* (*Desulfovibrio dechloroacetivorans*) able to both methylate Hg(II) and demethylate MeHg and strain *G200* (*Desulfovibrio desulfuricans*) only able to demethylate MeHg. Bacteria were grown in a synthetic medium in anaerobic conditions. They were spiked with 0, 0.1, 1 and 10 ppm $Hg(II)Cl_2$ (IHg) and MeHgCl (MeHg), and incubated for 24 h (growth parameters were followed in parallel to adjust the time of Hg exposure). Then, the culture was centrifuged, washed, blotted on Si_3N_4 membranes, and rapidly frozen in liquid ethane using the cryo-plunge freezer Leica EM GP. Bacteria deposits were then directly transferred to the cryostat in liquid nitrogen or freeze-dried using protocol developped for thin biological samples (several steps to progressively warm up from -120°C to 25°C during 11 hours). Several setups were tested (see results below).

Results:

1- Nano XRF mapping and Hg L_3 edge XANES at ambient temperature:

Since the cryo-set up was new and had not been yet entirely validated, we first tested our samples at ambient temperature to check that Hg was correctly detected and that Hg L_3 edge XANES could be collected (even we were aware that Hg speciation was probably modified by the sample preparation and by beam exposure). Mapping of Hg and essential metals (Zn, Fe, Ca) in bacteria was performed at 13 keV, just above the Hg L_3-edge with a flux of photons of 6. 10^8 ph.s^{-1}and a lateral beamsize of 95 nm x 86 nm while the fluorescence signal was collected using a one element SDD Vortex detector. Hg L_3-edge nano-XANES spectra were collected with the same detector from 12.234 to 12.434 keV. Hg was detected in *BerOc1* exposed to 10 and 1 ppm $HgCl_2$ (0.1 ppm exposure was not tested). XANES spectra collected on *BerOc*110 ppm were noisy (not shown) and we would have needed a long beamtime to obtain spectra with a good signal/noise ratio. Thus we decided to focus on nano-XRF mapping and to increase Hg detection using pink beam.

2- Nano XRF mapping at ambient temperature with pink beam:

XRF maps were performed at 17.5 keV, a flux of photons of 1.10^{11} ph.s^{-1}, and a beamsize of 56 nm x 56 nm. Fluorescence signal was collected with a 7-element detector. The methylating strain *BerOc1* and the non methylating strain *G200* exposed to 10 ppm $HgCl_2$ were analyzed. For *G200*, hot spots of Hg were mainly co-localized with Zn, preferentially inside the bacterial cell whereas Fe seemed more homogeneously distributed inside the cell. On the contrary, Ca was mainly located in the membrane (Fig. 1). For *BerOc1*, similar colocalization of Hg and Zn was observed, while Ca was mostly distributed in small grains along the microbe (Fig. 2).

BerOc1 exposed to 1 and 0.1 ppm IHg were analyzed but samples were not stable under beam exposure. It will be necessary to check if it is related to Hg amount. *BerOc1* exposed to 10 ppm MeHg seemed to have a different pattern: the colocalization of Hg and Zn was less obvious, and Hg was more homogeneously distributed inside the cell (Fig.3). Ca was distributed in the cell mambrane and in small grains inside the cell.

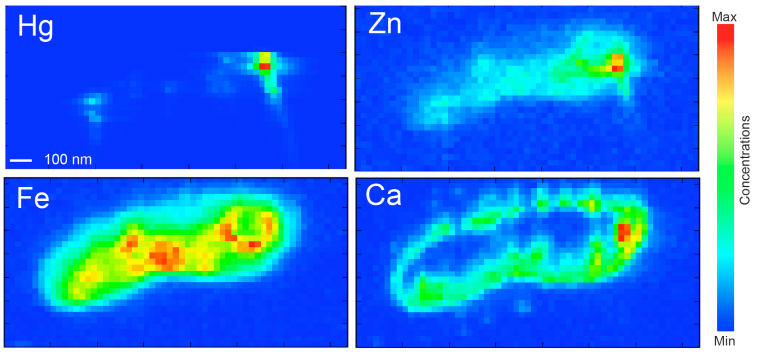

Fig.1: Elemental XRF maps of a *G200* bacterial cell exposed to 10 ppm IHg (E: 17.5 keV, beamsize: 56 nmx 56 nm, stepsize: 25 nm, counting time: 500ms/point, pink beam mode).

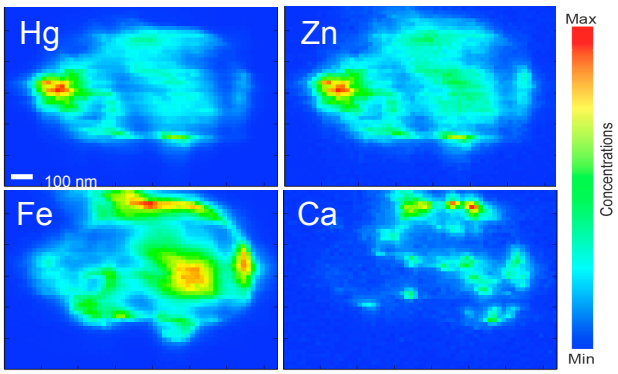

Fig.2: Elemental XRF maps of a *BerOc1* bacterial cell exposed to 10 ppm IHg (E: 17.5 keV, beamsize: 56 nmx 56 nm, stepsize: 25 nm, counting time: 500ms/point, pink beam mode).

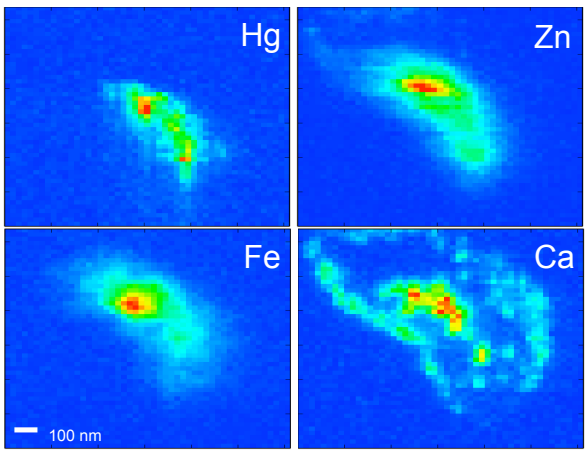

Fig.3: Elemental XRF maps of a *BerOc1* bacterial cell exposed to 10 ppm MHg (E: 17.5 keV, beamsize: 56 nmx 56 nm, stepsize: 25 nm, counting time: 500ms/point, pink beam mode).

3- Cryo-nano XRF mapping with pink beam:

Transfert and positionning of frozen bacteria deposited on Si_3N_4 membranes in the cryostat cooled with liquid nitrogen was first tested. This step was succesfull and we could rapidly mount the samples with a minimum of ice formation. The cryostat was then rapidly transferred to the beamline and connected to the liquid nitrogen supply to ensure a temperature of -150°C. This step was also successful. Cryo-nano-mapping was performed with the pink beam at 17.5 keV and the fluorescence signal was collected with the Silicon Drift Detector (the place taken by the cryostat did not allow to use the 7-element detector). Although coarse maps could be collected, it was not possible to obtain satisfactory maps with a nanometer size lateral resolution, because the samples were likely to slightly move in this set up, maybe because of the liquid nitrogen supply. This point should be delved.

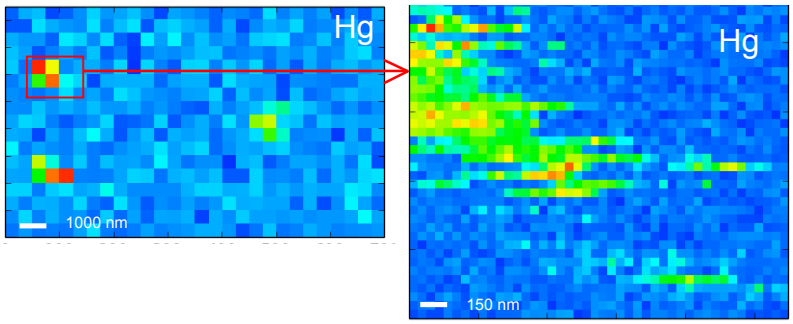

Fig.4: Hg XRF maps collected in cryo conditions. The coarse Hg map at the left shows various bacterial cells (stepsize: 500 nm) and the fine Hg map at the right shows a detailed image of the bacteria of the inset (stepsize: 50 nm). Image is of low quality probably due to sample movement during acquisition.

NB : There was a beamlost from 12h to 18h30 on October 1st.

Conclusion:

This experiment was expected to be challenging and accordingly, allowed us to point some difficulties in collecting data in cryogenic conditions. However this first run was encouraging since the cryo-sample preparation, the sample positioning in the cryostat, and the cryostat installation were successful. Interestingly, our bacterial samples are particularly suited to the improvement of measurements in cryogenic mode since they are easily found under the microscope, their size (~1 µm) is suited to nano-XRF mapping and Hg is detected. In a scientific point of view, nano-XRF mapping of freeze-dried bacteria allowed to draw some first conclusions of value. Firstly, in both methylating and not methylating strains, Hg is not preferentially located in the cell membrane of SRB but is heterogeneously found inside the cell. Secondly, Hg is generally colocated with Zn, which is a new insight and would be in agreement with the enter of Hg in the cell via Zn transporters as suggested by Schaefer *et al.* (2011; 2014). Finally, exposure to IHg and MeHg seems to result in different patterns of Hg association: Hg and Zn are clearly colocated in the case of IHg exposure while this is less obvious in the case of MeHg exposure.

These first results thus bring new insights and it is necessary to get deeper in the Hg pathways for both strains, for various Hg concentrations and chemical forms (IHg *vs* MeHg) and time of exposure to have a global view. For these reasons, we applied for additionnal beamtime. If measurements in cryogenic mode could be modified and improved before a next run, we are particularly interested in testing the setup, because we are convinced that cryo-preparation and cryo-measurements are necessary for XAS collection to avoid artefacts and speciation changes. If the setup can not be modified, we will collect nano-XRF maps on freeze-dried SRB exposed to various Hg concentrations, chemical forms and time of exposure (kinetics).

Schaefer et al. 2011. Active transport, substrate specificity, and methylation of Hg(II) in anaerobic bacteria. PNAS 108: 8714-8719.

Schaefer et al. 2014. Effect of divalent metals on Hg(II) uptake and methylation by bacteria. Environ. Sci. Technol. 48: 3007-3013.