Application of soft X-ray microscopy to environmental microbiology of hydrosphere

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Application of soft X-ray microscopy to environmental microbiology of hydrosphere

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Abstract. Microstructures of unprocessed filamentous cyanobacterium, *Pseudanabaena foetida* sp., producing a musty smell were observed using soft X-ray microscopy. Carbon-enriched structures and granules as well as oxygen-enriched granules which have been already reported were observed. Except for early log growth phase, the oxygen-enriched granules were observed. However, the carbon-enriched structures were observed throughout log growth phase. The result suggests there is a relationship between the oxygen-enriched granules and 2-methylisoborneol (2-MIB) productivity, since the 2-MIB productivity of each cell is increased depending on the culture period in log growth phase.

1. Introduction

In environmental microbiology of hydrosphere, the needs for observation of microbes and the mucous matter are high.

Lake Biwa is the most important water reservoir in Kyoto-Osaka-Kobe region in Japan. The high economic growth in the 1960s caused severe environmental problems in the lake such as eutrophication, losses in biodiversity, degradation of ecosystems. Although the water quality was gradually improved by cooperation with other stakeholders such as governments, local governments, citizens, the biota began to exhibit correspondingly drastic negative changes.

In 1969, a problem of musty smell in tap water has occurred due to sudden propagation of a certain green filamentous cyanobacterium. The filamentous cyanobacterium was reported as *Phormidium tenue* (Menegh.) Gomont and 2-methylisoborneol (2-MIB) was identified as a causative substance [1]. However, results of our soft X-ray microscopy (XM) observation suggested the necessity of reidentifying of it [2, 3]. In 2016, as a result of morphological and genetical analysis, the cyanobacterium was proposed as a new planktonic species producing 2–MIB: *Pseudanabaena foetida* Niiyama, Tuji *et* Ichise sp. nov. [4]. Now, the study how the microstructure and culturing conditions are related to the 2-MIB productivity is proceeding. The aim of this study is to elucidate the microstructure related to the 2-MIB productivity of *P. foetida* sp. using the XM.

2. Materials and methods

2.1. Phytoplankton Material

P. foetida sp. was collected from Lake Biwa and subcultured. The culture was maintained on CT medium (pH 8.0) under a 12 h light/12 h dark cycle at 20 °C. The cultures were illuminated with fluorescent lamps, which provided about 85 μmol photons m^{-2} s^{-1} of photosynthetically active radiation.

2.2. X-ray microscopy

Phytoplankton was observed by soft XM using the BL-12 microscope at the SR Center of Ritsumeikan University [5, 6]. The microscope was equipped with Fresnel zone plate condenser and objective with 53.7 nm and 36 nm outer zone widths respectively. The images were recorded using an X-ray CCD camera (C4880-21-24WD: Hamamatsu Photonics K.K.). The achievable spatial resolution was approximately 70 nm based on the knife-edge estimation (20 - 80%) at 517 eV (see e.g. [7]).

Projection images were collected using exposure times ranging from 60 to 120 s. Background images were also collected and each image was divided by the background image. Cryogenic imaging is allowed by using the indirect contact cryogenic system which enables cooling to around -160 °C without frost [8].

2.3. Sample preparation and observation

For 2D imaging, the cells suspension dropped onto a 100 nm thickness silicon nitride membrane window (Silson Ltd.). After air-dried, the samples were observed by XM at 620 eV and 532 eV at room temperature.

For 3D imaging, the cell suspension was injected into a glass capillary tube with a fine tip that was created by using a puller, PC-10 (Narishige). For acquisition of whole data set, 75 X-ray projection images were acquired with rotating the sample 4 degree each (180 degree rotation). I_0 images were acquired every 5 projections to compensate decay of beam current. The cross sectional images were reconstructed with convolution back projection algorithm. The reconstructed volumetric data was visualized using VGStudio MAX 2.0 (Volume Graphics GmbH). The spatial resolution of the cross sectional image was approximately 160 nm based on the edge response (20 - 80%) of the glass capillary at 517 eV.

3. Results and discussion

Figure 1 shows XM images of air-dried *P. foetida*. Although filamentous bacteria are often surrounded with sheaths, *P. foetida* cells were not surrounded with sheaths. Any metabolite-like agar or gelatin were not also observed. Several granules were observed in the cells 4 weeks after inoculation, log growth phase (Fig.1 a). As reported previously, the granules disappear at 532 eV. This result indicates that the granules are oxygen-enriched structures such as polyphosphate granules. A cell 1 week after inoculation, early log growth phase, showed interesting structures which have been not observed under previous XM observation (Fig.1 b). In the middle of the cell, a boundary line is seen. From the boundary line, the structures extend to both ends of a cell. The structures are almost linearly symmetrical with the boundary line. It is noteworthy that the structures appear not only in 620 eV image but also in 532 eV image. This result indicates that a dividing cell has been observed and the structures are carbon-enriched structures such as nucleoid or prokaryotic cytoskeleton [9].

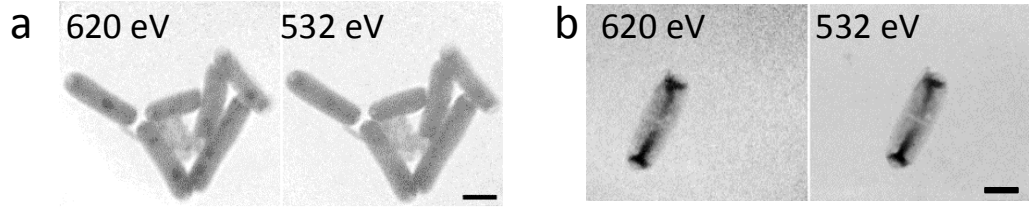

Figure 1. XM images of air-dried *P. foetida*. Images of cells 4 weeks after inoculation, log growth phase, were taken at 620 eV and at 532 eV (a). Images of a cell 1 week after inoculation, early log growth phase, were taken at 620 eV and at 532 eV (b). Exposure time was 120 s. Scale bar is 2 μm.

The carbon-enriched structures were observed throughout log growth phase. However, few oxygen-enriched granules were observed in early log growth phase. In log growth phase, 2-MIB productivity of each cell is increased depending on the period of its culture. The result suggests that the oxygen-enriched structures such as polyphosphate granule relate to 2-MIB productivity and the carbon-enriched structures such as nucleoid or prokaryotic cytoskeleton are not related to it.

To verify the oxygen-enriched structures, distribution of oxygen and oxygen K-edge XANES spectra were collected by the scanning transmission X-ray microscope (STXM) at UVSOR BL4U [10, 11]. Figure 2 shows STXM images and a distribution of oxygen in *P. foetida* grown to early stationary phase. Four oxygen-accumulated granules consisting of large and small sizes were observed. Although absorption spectra were noisy, a spectrum from the granule obviously exhibited a pre-peak which is different from that of organic carbonyl group (C=O) at about 532 eV. To identify the granules, we intend to promote a more detailed analysis.

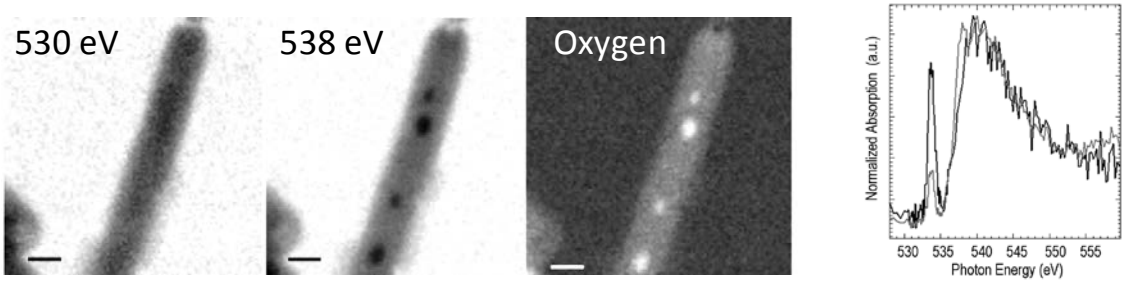

Figure 2. STXM images and oxygen K-edge XANES spectra. STXM images and distribution of oxygen in the air-dried *P. foetida* cells grown to log growth phase. Scale bar is 1 μm. Oxygen K-edge XANES spectra of cytoplasm (black line) and granule (grey line).

Figure 3 shows 3D reconstructed tomograms and 2D slice images of *P. foetida* cells grown to late log growth phase. Many rod-shaped bacteria without sheath were observed. In the cells, heterogeneous X-ray absorption was seen, whereas carbon-enriched granules were not seen. These results are the same that in air-dried 2D observation at 532 eV (Fig.1 a). However, *P. foetida* cells grown to death phase contained several carbon-enriched granules (Fig.4). The cells shrank because of radiation damage during repeated X-ray exposure. However three different granules were inside different cells. Although this result indicates that the granules are carbon-enriched structures, candidate prokaryotic organelles corresponding to the granules have not been assigned yet.

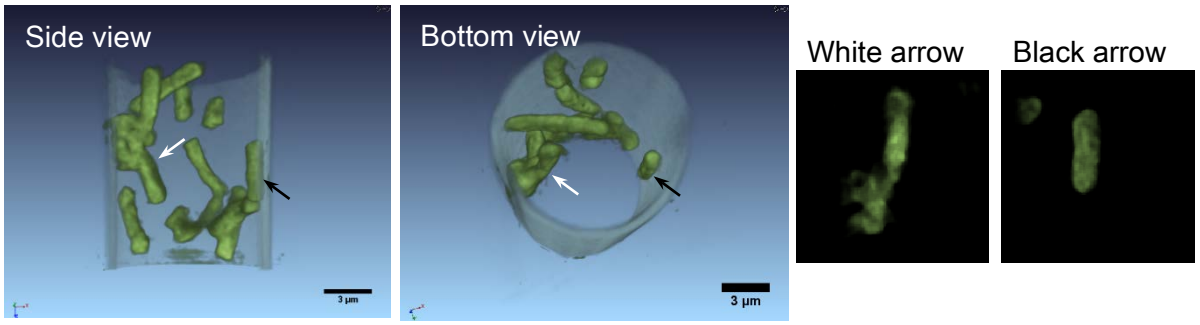

Figure 3. Cryo-XM images of *P. foetida* grown to late log growth phase in a hydrated state at -110 °C. Three-dimensional rendering images (side and bottom views) and longitudinal section images of cells (white and black arrows). Images were taken at 532 eV and exposure time was 60 s.

4. Conclusions

To investigate how the microstructure is related to the 2-MIB productivity, unprocessed *P. foetida* cells were observed by using soft XM. The *P. foetida* cells were not surrounded with sheath. Carbon-enriched

structures such as nucleoid or prokaryotic cytoskeleton were observed throughout log growth phase although few oxygen-enriched granules such as polyphosphate granule were observed in early log growth phase. In addition, carbon-enriched granules were observed in cells grown to death phase although any candidate prokaryotic organelles corresponding to the granules have not been assigned yet.

During log growth phase, 2-MIB productivity of each cell is increased depending on the period of its culture. The result suggests that the oxygen-enriched structures relate to 2-MIB productivity and the carbon-enriched structures do not relate to it.

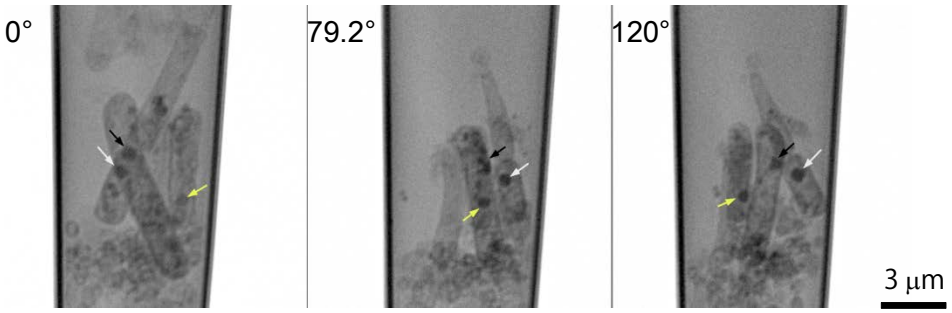

Figure 4. XM images of *P. foetida* grown to death phase in a hydrated state at room temperature. Image was taken at 517 eV and each exposure time was 120 s. Three different granules (black, white and yellow arrows) were inside different cells.

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