Soft x-ray imaging of intracellular granules of filamentous cyanobacterium generating musty smell in Lake Biwa

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Abstract. A planktonic blue-green algae, which are currently identified as Phormidium tenue, was observed by a soft x-ray microscopy (XM) for comparing a musty smell generating green strain (PTG) and a non-smell brown strain (PTB). By XM, cells were clearly imaged, and several intracellular granules which could not be observed under a light microscope were visualized. The diameter of granules was about 0.5-1 μm, and one or a few granules were seen in a cell. XM analyses showed that width of cells and sizes of intracellular granules were quite different between PTG and PTB strains. To study the granules observed by XM, transmission in more detail, transmission electron microscopy (TEM) and indirect fluorescent-antibody technique (IFA) were applied. By TEM, carboxysomes, thylakoids and polyphosphate granules were observed. IFA showed the presence of carboxysomes. Results lead to the conclusion that intracellular granules observed under XM are carboxysomes or polyphosphate granules. These results demonstrate that soft XM is effective for analyzing fine structures of small organisms such as cyanobacterium, and for discriminating the strains which generates musty smells from others.

1. Introduction
Since 1969, musty odor problems have frequently occurred in the water supply at Lake Biwa. 2-methylisoborneol (MIB) and geosmin were identified as the causative substances. At present, three species of planktonic blue-green algae from Lake Biwa have been identified as organisms playing important roles in such problems. From the unialgal culture of each alga, Anabaena macrpospora Klebahn was shown to produce geosmin, and both Phormidium tenue (Meneghini) Gomont and Oscillatoria tenuis Agardh to produce MIB [1].
A planktonic blue-green algae, which is currently identified as *P. tenue*, is a small filamentous cyanobacterium with a firm sheath, and the trichome is consisted of cells 1.0–1.6 μm in width, and 2.5–5 μm in length. The sheath is too thin to be visualized by a light microscope (LM). *P. tenis* is a well known MIB producer and is closely associated with the occurrence of musty odor in Lake Biwa. The presence of two-color plankton, green and brown species, have been reported [2]. Some green species produces MIB, and some green species do not produce MIB [2]. On the other hand, all brown species do not produce MIB [2]. Because of a small cell size, it is difficult to know whether there are morphological differences between green strain (PTG) and brown strain (BTB) or between musty smell generating and non-generating strains under the light microscopic examination. Self fluorescence microscopy is a unique method to discriminate such strains from others [2]. However, it is reported that application of this method is restricted to some specific strains.

Soft x-ray microscope (XM) is a powerful tool to investigate whole cells up to 10 μm thickness with much higher resolution than LM. We observed the cyanobacterium by a soft XM to image fine structure in the cells, and compared the morphological difference between the strains. To identify an intracellular structure which was observed by XM, transmission electron microscopy (TEM) and indirect fluorescent-antibody technique (IFA) were also applied.

### 2. Experimental

#### 2.1. X-ray microscopy (XM)

Suspension of the laboratory-cultured musty smell generating green *P. tenue* strain (PTG) and musty smell non-generating brown strain (PTB) (see Figure 1) was dropped onto a polyimide thin film and air-dried [3]. XM images were acquired by using a soft X-ray microscopy beamline (BL-12) at the SR Center of Ritsumeikan University. Two wavelengths, 2.0 nm and 2.4 nm (below and above the wavelength of the oxygen K-edge threshold), were used for imaging. A resolution at 2.0 nm and 2.4 nm was ca. 80 nm and 88 nm, respectively, judging from the knife-edge estimation (20–80 %).

#### 2.2. Transmission electron microscopy (TEM)

Laboratory-cultured PTG suspension was dropped onto a polyimide thin film and air-dried. After fixation with glutaraldehyde and subsequently with osmium tetroxide, cells were embedded in epon. Thin sections are doubly stained with uranium and lead. TEM images were acquired by using Hitachi H-7600 TEM operating at 80 kV accelerating voltage.

#### 2.3. Indirect fluorescent-antibody technique (IFA)

Laboratory-cultured PTB suspension was dropped onto a polyimide thin film and air-dried. Cells were fixed in pre-chilled 100 % methanol were rinsed in PBS and were incubated for 60 min at 4°C in blocking solution containing PBS and 5 % bovine serum albumin (BSA). Cells were then incubated with a polyclonal anti-RuBisCO antibody (Agrisera, Prod. ID AS03 037) diluted 1:500 in blocking solution overnight at 4°C. Cells were washed in PBS and then incubated with an anti-rabbit Oregon green conjugated antibody (Invitrogen) diluted 1:300 in blocking solution for 1 hr at 30°C. Cells were washed in PBS and imaged [4]. IFA image was acquired by using Zeiss Axiovert 200M epifluorescence microscope.

### 3. Results and discussion

Figure 2 shows x-ray microscopic images of PTG and PTB. Each cell was clearly observed. Granules were also clearly recognized. All granules were not observed by 2.4 nm observations [5]. These granules were also observed in wet condition in our previous XM observations. Thus, we reconfirm...
that granules were observed both in dry and wet condition [5, 6]. Results also show that oxygen is an important constituent element of the granule. Cellular size of PTG was larger than that of PTB. Using x-ray micrographs, average size of cells were estimated. The average width of PTG and PTB was 1.35 \( \mu m \) and 1.08 \( \mu m \), respectively. Although the number of intracellular granules is about the same (~2 / cell), the size distribution of the granules was quite different between PTG and PTB. The average diameter of PTG and PTB was 0.77 \( \mu m \) and 0.42 \( \mu m \), respectively.

To investigate the granules in more depth, TEM and IFA were applied to the PTG cells. Figure 3 shows a TEM image of a representative PTG cell on the ultra-thin section. Thylakoid membranes appeared as thin layers around the cell in cross section. The cell included carboxysomes and polyphosphate body. Carboxysomes are spatially ordered in a linear fashion in the cell. Carboxysomes are bacterial microcompartments that contain enzymes, RuBisCO, involved in carbon fixation. Next, IFA was applied to visualize the carboxysomes using anti-RuBisCO antibody. By the IFA, the carboxysomes were also spatially ordered in a linear fashion (Figure 4).

These results show that soft XM is effective for analyzing fine structure of small organisms such as cyanobacterium, and discriminating the strains which generates musty smells from others.

![Figure 2. XM images of PTG (A) and PTB (B). Scale bar is 2 \( \mu m \). Observation wavelength was 2.0 nm. Each exposure time was 120 s.](image2)

![Figure 3. TEM images of PTG. Scale bar is 500 nm. Green area: thylakoid membranes, membrane system where the fully functional electron transfer chains of photosynthesis and respiration reside. Yellow area, carboxysomes, bacterial microcompartments that contain enzymes involved in carbon fixation. Blue area: polyphosphate body, intracellular nutrient storage granules.](image3)

![Figure 4. Micrographs of PTB. Left image is XM. Right image is IFA image of the corresponding cells. Scale bar is 5 \( \mu m \).](image4)

4. Conclusions
We observed a small filamentous cyanobacterium, *P. tenue*, by the soft XM for comparing a musty smell generating strain and a musty smell non-producing strain.

Experimental results led to the following conclusions: (1) soft XM showed morphological difference between a musty smell generating strain (PGB) and a musty smell non-producing strain.
(PGB). (2) Soft XM is one of optimal tools to distinguish different cyanobacteria strains such as PTG and PTB. (3) TEM and IFA showed that intracellular granules observed under XM may be carboxysomes or polyphosphate granules.

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