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Worm, *Oligobrachia mashikoi*, Frenulate,  
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## Detection of Sulfur Using an X-ray Analytical Microscope from the Trophosome of a Beard Worm, *Oligobrachia mashikoi*, Frenulate, Siboglinidae

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### Abstract

In one species of Frenulata which has neither a mouth nor a digestive tract, element analysis of the part including the trophosome, in which chemosynthetic bacteria are harbored, and of a non-trophosome not including the bacteria was conducted using an X-ray analytical microscope. As a result, signals of sulfur were detected predominantly from the part including the trophosome.

**Key Words:** Siboglinidae polychaetes, *Oligobrachia maoshikoi*, X-ray analytical microscope, sulfur, chemosynthetic bacteria, trophosome

### I. Introduction

The polychaetes of Siboglinidae in Annelida are divided into the following three groups: Frenulata (beard worm), Vestimentifera (tube worm), and Monilifera (*Sclerolinum*). Although they have neither a mouth nor a digestive tract, they harbor chemosynthetic bacteria in their body (Bright and Giere, 2005; Southward *et al.*, 2005). The symbiosis of the siboglinid polychaetes with bacteria was confirmed by enzyme histochemistry for the first time in tube worms that inhabit hydrothermal vents. It was suggested that the bacteria were sulfur-oxidizing ones (Cavanaugh *et al.*, 1981). In the trophosome of tube worms, the existence of sulfur has been investigated in detail. In *Lamellibrachia* sp. inhabiting the sea bottom, where hydrogen sulfide and

methane seep, there is variation in the morphology of symbiotic bacteria (Naganuma *et al.*, 1997). Using an X-ray microanalyzer, the sulfur content has been shown to vary among bacteria of different morphology. In addition, Pflugfelder *et al.* (2005) noted variation in the color of the trophosome in *Riftia pachyptila*. Using an electronic energy-loss spectroscopy, they found that the sulfur content of synthetic bacteria was different in individuals of different color.

Also in Frenulata, symbiotic bacteria are regarded as sulfur-oxidizing (Southward *et al.*, 1986; Schulze and Halanych, 2003). In this group, however, there is no direct evidence of which symbiotic bacteria are sulfur-oxidizing. In this study, we tried to detect the existence of sulfur from the trophosome of *Oligobrachia mashikoi* using an X-ray analytical microscope.

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## II. Materials and Methods

*O. mashikoi* was collected from the sea bottom at a 25 m depth in Tsukumo Bay, Noto Peninsula, Ishikawa Prefecture, using a previously described method (Sasayama *et al.*, 2003). They were preserved in 70% ethanol after fixation with a 4% paraformaldehyde PBS solution for about 12 hours.

The samples were taken to the Horiba, Ltd., Technical Plaza (Tokyo), where an attempt was made to detect sulfur from the trophosome using an X-ray analytical microscope (XGT-5000, Horiba, Ltd., Kyoto), which is an advanced type of X-ray fluorescence spectrometer. The principle of the X-ray analytical microscope is to detect the fluorescence X-rays excited from materials irradiated with X-rays, to identify elements by the specific wavelength of the fluorescence X-ray of each element, and to quantify the element by its wave strength. Every element from sodium to uranium can be detected at the same time.

Each sample, both with and without the trophosome, was about 1.5cm long. The samples were supported with a mylar membrane to prevent evaporation of the 70% alcohol and placed on a sample stage. We observed the samples through a microscope display and

decided which parts should be irradiated by X-rays. The diameter of the sample containing a trophosome was 0.66 mm. Five spots upon which X-rays were perpendicularly irradiated were identified (Fig.1a). The diameter of the sample not containing the trophosome was 0.73 mm, and seven spots were identified (Fig.1b).

In this study, the diameter of the X-ray guide tube was set at 10  $\mu$ m in order to examine the smallest spots possible. The electric current and the voltage were set to 1 mA and 50kV, respectively. The irradiation time on any one spot was set to 100 seconds. The pulse processing time was adjusted for the P3 level to efficiently detect only major elements. These conditions can detect only masses of sulfur element, not sulfur included in an amino acid. The quantity of elements was calculated using the fundamental parameter method.

After observation with this microscope, the samples were remade into paraffin sections of 10  $\mu$ m in thickness by a routine method and stained with hematoxylin and eosin. This procedure is necessary to identify the tissue inside of the X-ray-irradiated spot, as shown in Fig.1.

## III. Results

### 1) Part containing the trophosome

In Figure 2, cross section of a specimen observed under an X-ray analytical microscope and X-ray irradiated parts are shown; it corresponds to spots 1 to 5 in Figure 1a.

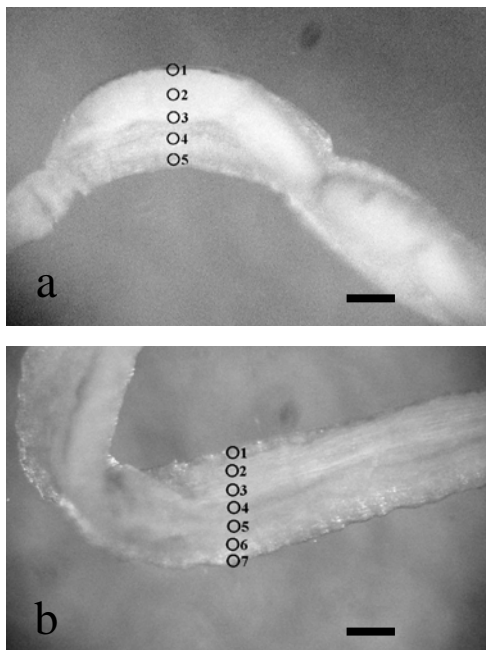


Fig.1a Part of the whole body containing the trophosome. Bar = 0.3mm. 1b Part of the whole body not containing the trophosome. Bar = 0.3mm. The spots and numbers correspond to the parts irradiated with X-rays.

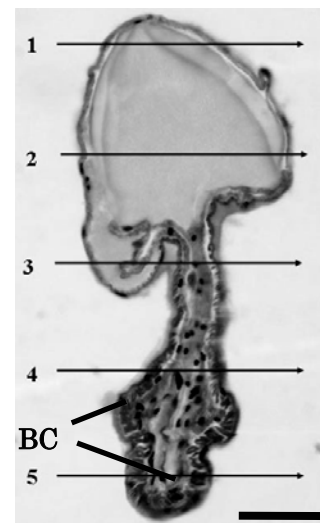


Fig.2 X-ray-irradiated parts (1 to 5) in the specimen containing the trophosome. DV: dorsal vessel; BC: bacteriocytes. Bar = 0.1 mm.

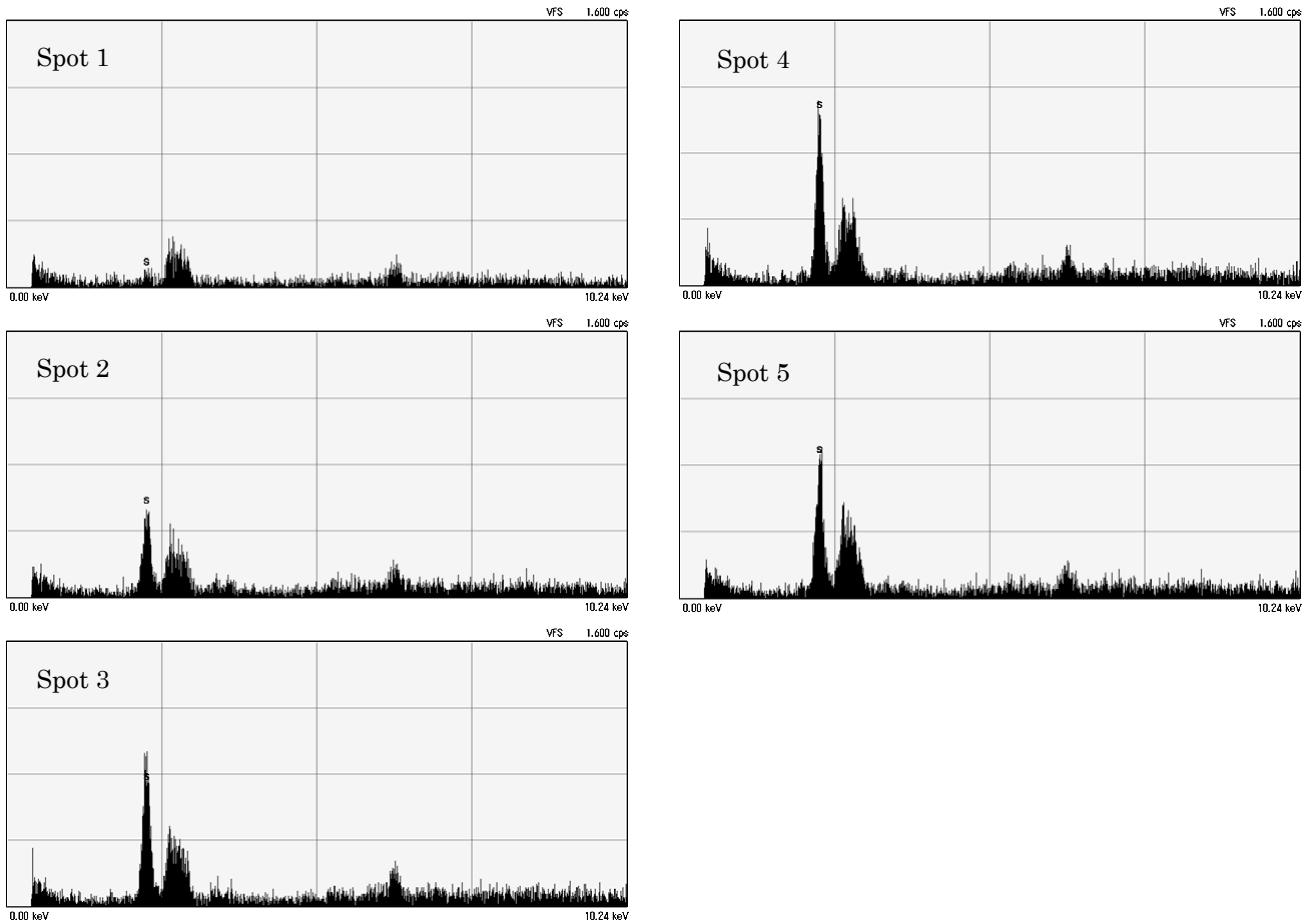


Fig.3 Element distribution patterns obtained in spots 1 to 5.

Figure 3 shows element spectrum from irradiation spots 1 to 5.

As is evident in Figure 3, only sulfur was detected, and the other elements were equal to or less than the detection limit. The broad peak observed always on the right side of the sulfur peak is rhodium, which is a constituent element of an X-ray gun used for the generation of X-rays. Therefore, it is not a true ingredient of the specimen.

At spot 1, when the X-rays irradiated the upper end of the dorsal blood vessel, no sulfur was detected. From spot 2, however, in which the X-rays irradiated through the dorsal blood vessel, sulfur was detected. The value of the X-ray intensity was 6.90 cps/mA. In spot 3, the value was 11.90 cps/mA. Furthermore, a higher level of sulfur was detected from spot 4, which is the central part of the trophosome. The value was 13.84 cps/mA. Even at spot 5, which is the basal part of the trophosome, the sulfur density still showed a high value, 11.30 cps/mA.

## 2) Part not including the trophosome

In Figure 4, the part inside of the specimen that was X-ray-irradiated is shown; it corresponds to spots 1 to 7 in Figure 1b.

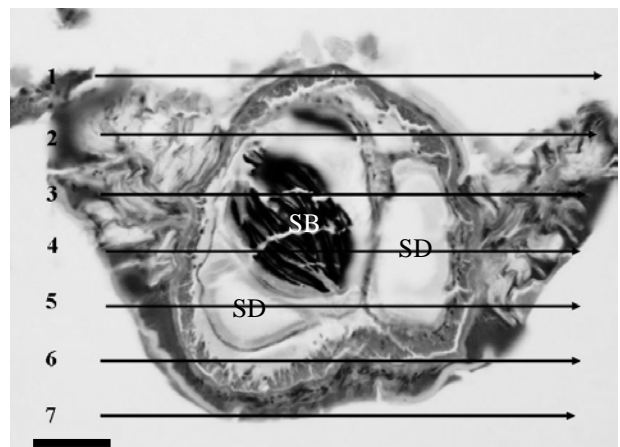


Fig.4 X-ray-irradiated parts in a specimen not including the trophosome. Bar = 0.1 mm. SB: sperm bundles; SD: sperm duct

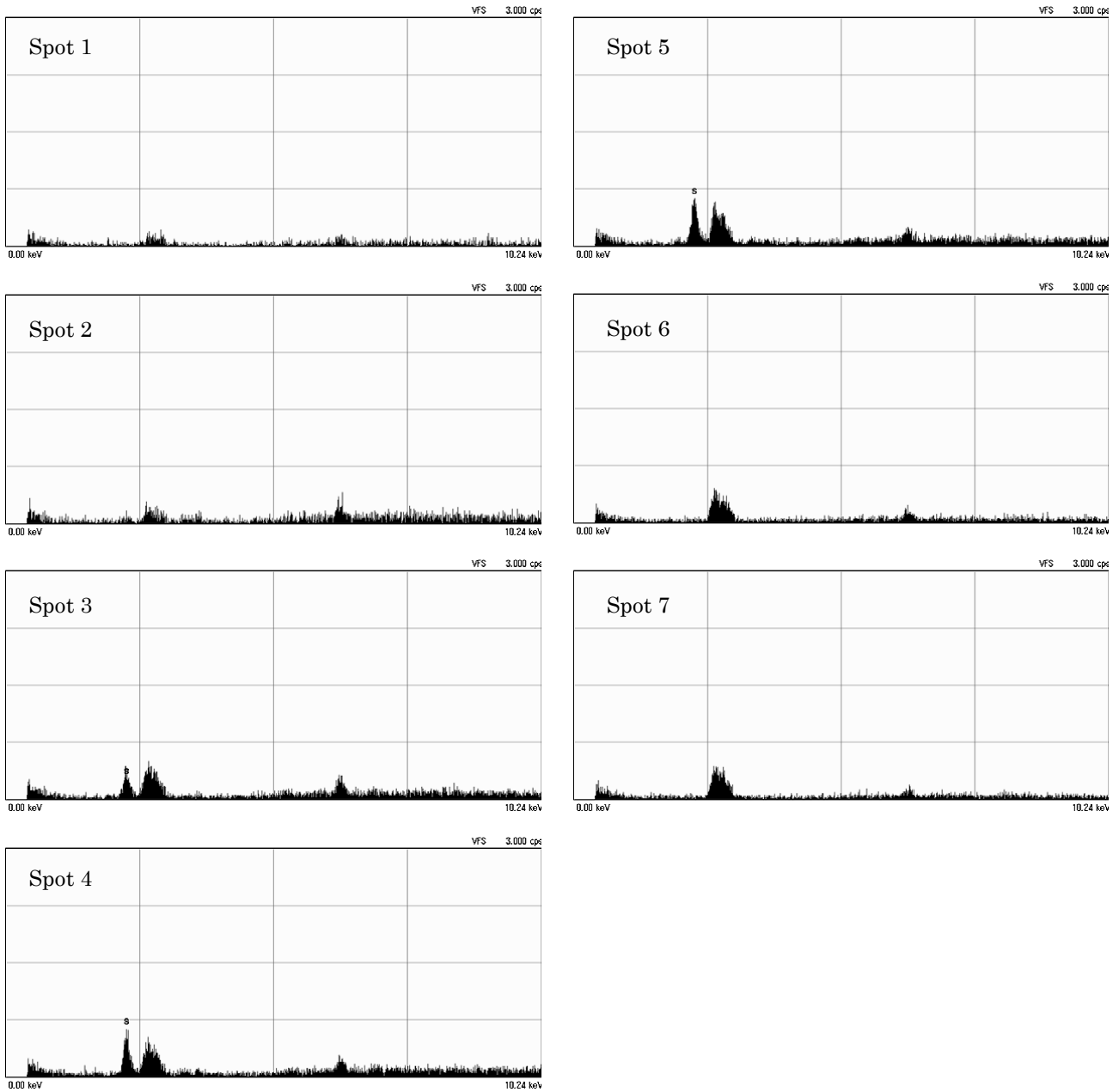


Fig.5 Element distribution patterns obtained in the part not containing the trophosome in spots 1 to 7.

Figure 5 shows element detection patterns from irradiation spots 1 to 7.

The non-trophosome part included a testis. In spots 3, 4, and 5, sulfur was detected. Their X-ray intensity levels were, however, rather low, *i.e.*, 4.16, 7.41, and 7.91 cps/mA, respectively.

#### IV. Discussion

Recently, we analyzed the mud from the sea bottom of the bay inhabited by *O. mashikoi* and detected

hydrogen sulfide, although the concentration was not high (Sasayama *et al.*, 2007). On the other hand, we succeeded in the analysis of the three-dimensional structure of the extra-cellular hemoglobin of this species (Numoto *et al.*, 2005). In this molecule, there was a non-paired cysteine residue, and it was suggested that the sulfur of the hydrogen sulfide was bound by this residue

In the part containing the trophosome, a peak of the sulfur was at first detected in spot 2, where X-rays irradiated the central part of the dorsal vessel. This fact appears to reflect the existence of large quantities of

hemoglobin bound with sulfur. In addition, in the part not containing the trophosome, sulfur was detected at spots 3, 4, and 5. In these regions, no large blood vessels were found. However, considering that the peak height is similar to that in spot 2 of the dorsal vessel in the trophosome, hemoglobin bound with sulfur might be present in the capillary blood vessels. Furthermore, in the part containing the trophosome, high peaks of sulfur were obtained at spots 3 and 4, where X-rays irradiated the trophosome. The peak of sulfur was still high at spot 5 of the basal part of the trophosome. Although the existence of symbiotic bacteria is not clear in the resolution of the light microscope used in this study, it is known that, in so-called bacteriocytes, which harbor chemosynthetic bacteria, there are specific lysosomal granules (Southward, 1993; Deguchi *et al.*, 2007). Therefore, the regions in which such granules can be recognized must be bacteriocytes (Fig.6). Consequently, it is probable that the chemosynthetic bacteria living in *O. mashikoi* are sulfur-oxidizing bacteria.

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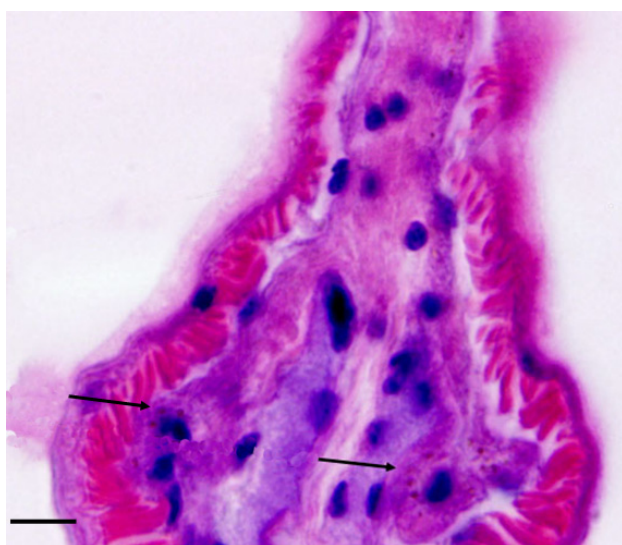


Fig. 6 A photograph showing the presence of bacteriocytes (arrows) in the trophosome, which include lysosomes (red-colored small granules) around a nucleus (dark-bluish large granule). Bar = 0.02mm.

the microscope and provided a thorough explanation of the procedure.

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## Siboglinidae科, Frenulate類のマシコヒゲムシ, *Oligobrachia mashikioi*の 栄養体から, X線分析顕微鏡を用いてイオウ元素の検出

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### 要 旨

ヒゲムシ類は口も消化管も無い。そのかわり, 体内に化学合成細菌を共生させ, それが生産する炭水化物をもらい生きている。一方, 共生細菌は, これまで 16SrDNA の塩基配列や生息環境に硫化水素が存在することなどより, 酸化エネルギー基質としてイオウを要求するイオウ酸化細菌であると考えられてきた。本研究ではマシコヒゲムシを用いて, 細菌を共生させている栄養体を含む部分と, 栄養体を含まない部分を X 線分析顕微鏡で元素分析を行った。その結果, 共生細菌が含まれる部分からイオウ元素が検出された。このことは, 宿主が環境にある硫化水素を栄養体に棲む共生細菌まで血流によって運び, 細菌にそれをわたしている可能性を示唆している。また直接, イオウが検出されたことにより, この細菌はイオウ酸化細菌であることをさらに補強するものである。

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