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Article

# High-voltage electron microscopy tomography and structome analysis of unique spiral bacteria from the deep sea

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## Abstract

Structome analysis is a useful tool for identification of unknown microorganisms that cannot be cultured. In 2012, we discovered a unique deep-sea microorganism with a cell structure intermediate between those of prokaryotes and eukaryotes and described its features using freeze-substitution electron microscopy and structome analysis (quantitative and three-dimensional structural analysis of a whole cell at the electron microscopic level). We named it *Myojin parakaryote*. Here we describe, using serial ultrathin sectioning and high-voltage electron microscopy tomography of freeze-substituted specimens, the structome analysis and 3D reconstruction of another unique spiral bacteria, found in the deep sea off the coast of Japan. The bacteria, which is named as 'Myojin spiral bacteria' after the discovery location and their morphology, had a total length of 1.768 ± 0.478 μm and a total diameter of 0.445 ± 0.050 μm, and showed either clockwise or counter-clockwise spiral. The cells had a cell surface membrane, thick fibrous layer, ribosomes and inner fibrous structures (most likely DNA). They had no flagella. The bacteria had 322 ± 119 ribosomes per cell. This ribosome number is only 1.2% of that of *Escherichia coli* and 19.3% of *Mycobacterium tuberculosis* and may reflect a very slow growth rate of this organism in the deep sea.

**Key words:** deep-sea, freeze-substitution fixation, high-voltage electron microscopy, spiral bacteria, structome, tomography

## Introduction

In 2012, we discovered a unique microorganism that has intermediate cellular structures between prokaryotes and eukaryotes, and named it the 'Myojin parakaryote' after the discovery location and its intermediate morphology [1]. From our observations using ultrathin sections of freeze-substituted specimens with electron microscopy, it became apparent that there are many other strange microorganisms in the deep sea [2,3]. Among them, unique spiral bacteria were conspicuous and abundant in the deep-sea specimens. We undertook structome analysis of these spiral bacteria ('structome' was defined as the 'quantitative and three-dimensional structural information of a whole cell at electron microscopic level' [4,5]). Because the spiral bacteria had similar size and similar morphological features in both external and internal structures, the population of the spiral bacteria may belong to the same species and can be named as 'Myojin spiral bacteria (MSB)' after the discovery location and its unique morphology. Here we report the structome of the MSB and whole 3D images obtained by serial ultrathin sectioning and high-voltage electron microscopy (HVEM) tomography.

## Materials and methods

### Sample collection, specimen preparation and structome analysis of cells

Samples were collected from hydrothermal vents at the Myojin Knoll (32°08.0′ N, 139°51.0′ E) off the coast of Japan at a depth of 1240 m in May 2010 [1]. Small invertebrates, such as polychaetes, and their associated microorganisms were collected and fixed with 2.5% glutaraldehyde. They were brought to the laboratory at Chiba University, snap-frozen, freeze-substituted [6,7] and embedded in an epoxy resin. Serial ultrathin sections of 70-nm thickness were cut, picked up on slit grids [8] and observed in a JEM-1400 electron microscope (JEOL, Tokyo, Japan) at 10 000–25 000 nominal magnifications.

Structome analysis of the spiral bacteria using serial sections was undertaken on six individuals using micrographs of six to eight complete serial sections for each individual. Images analyses were performed using Microsoft PowerPoint:mac 2011 and Fiji (Image J http://imagej.nih.gov/ij/) software [9,10]. Briefly, because the whole cell body was encompassed with several serial ultrathin sections, cell length was calculated by measuring the length of the line drawn between two ends of each cell on the superimposed images inserted in the power point slide where the reference line drawn along the scale on the image was measured. The total length and total diameter of the cell body, the diameter of the cell body and the pitch of the cell body were measured as a pixel value using the line selection menu in the ImageJ/Fiji window as well as a scale bar recorded on the same image. Measured pixel values were converted to μm or nm according to the measured pixel value of the scale bar on the corresponding images.

The cross-sectional area of each cell was determined using the 'Measure' command in the 'Analyze' menu of ImageJ/Fiji by tracing the outermost membrane using the polygonal selection menu in the ImageJ window (see the supplementary data online, Fig. s1) and converting the area result above into $\mu m^2$ by multiplying the square of the ratio of scale (nm) on the same image by its pixel value. Then, the volume ($\mu m^3$) of each cell was calculated as the cumulative volume of the parts having the cell's cross-sectional area as the base and the section thickness (0.070 μm) as the height.

Ribosomes, recognized as electron-dense particles with 10–20 nm diameters in the cytoplasm of the cell cross-section in each serial ultrathin section, were enumerated using the 'Multi-point Tool' in ImageJ/Fiji (see the supplementary data online, Fig. s1). The total number of ribosomes in each cell and the number of ribosomes per 0.1 $\mu m^3$ of cytoplasm were calculated based on the volume of each cell determined as described above.

### HVEM tomography

It was not possible to determine by a conventional 100-kV electron microscope whether thick (1–2.5 μm) sections contain the MSB or not, because the electron beam does not penetrate such thick sections. We therefore prepared normal ultrathin sections and thick sections, alternately. The ultrathin sections were stained with uranyl acetate and lead citrate [11], covered with Super support film (Nisshin EM, Tokyo), and observed with a 100-kV electron microscope (JEM-1400) at Chiba University. On the other hand, thick sections beyond 1 μm were stained by saturated uranyl acetate solution at 60°C for 20 min, lead citrate at 60°C for 10 min, and mounted on double 75-mesh grids. Only thick sections that were pre-evaluated with the neighboring ultrathin sections to include the MSB (hence the thick section certainly contained the MSB) were brought to HVEM facilities at Okazaki and Nagoya to examine.

For HVEM at National Institute for Physiological Sciences (Okazaki, Japan), a tomographic tilt series of 1.0 μm thick sections were imaged over an angular range between +60° and −60° with tilt increment steps of 2° using an accelerating voltage of 1000 kV (Hitachi H-1250 M). The nominal magnification was ×2000, and a conventional TEM mode was used. Tomographic reconstructions and segmentations were performed using the IMOD software [12].

For HVEM at Nagoya University (Nagoya, Japan), a tomographic tilt series of 2.5 μm thick sections were photographed over an angular range between +60° and −60° with increments of 2° at 1000 kV with JEM-1000K RS (JEOL) at ×100 000 magnification [13]. Using a STEM (scanning transmission electron microscopy) mode camera, it was possible to focus whole objects even at a high angle of the sections using the dynamic focusing system. Thus, clear images could be obtained at any angles and at high magnifications in this instrument [14]. Tomographic reconstructions were obtained with TEMography (System In Frontier Inc.).

## Results

### Cell structure of the MSB

The cell structure was examined by serial ultrathin sectioning (Figs. 1 and 2, and the supplementary data online, Figs. s1–s5) and HVEM tomography (Figs. 3 and 4, and the supplementary data online, Movies 1–4).

MSB had a spiral shape (Figs. 1–4) and consisted of a cell surface membrane (9 nm thick), thick fibrous layer, ribosomes and inner fibrous structures (most likely DNA) (Figs. 1 and 2, and the supplementary data online, Fig. s1). There were no flagella in the MSB (Figs. 1–4, and the supplementary data online, Figs. s1–s5).

### 3D Reconstructions of the MSB by HVEM tomography

Because the MSB are small, it was difficult to make 3D images from the serial sections (see the supplementary data online, Movie 5). To visualize the whole morphology of MSB, it was necessary to observe thick (1–2.5 μm) sections that contain whole MSB by HVEM and take tomography of the MSB.

Figure 3 shows reconstructed 3D images obtained by Hitachi HVEM tomography at Okazaki (see the supplementary data online, Movies 1 and 2). Figure 4 shows reconstructed 3D images obtained by JEOL HVEM

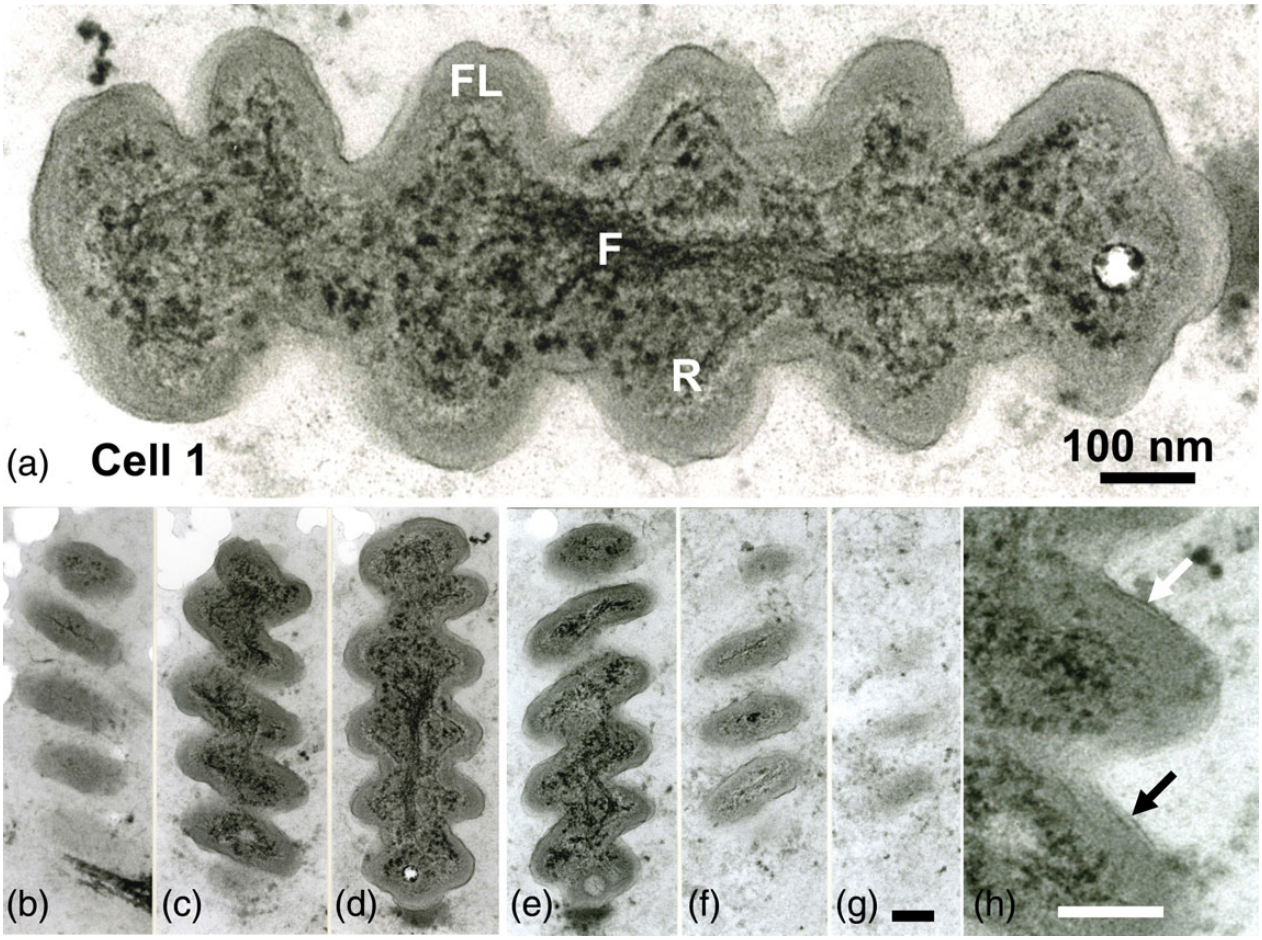


**Fig. 1.** Ultrathin sections of the freeze-substituted myojin spiral bacteria (MSB). (a) High magnification of the MSB (Cell 1). F, inner fibrous structures; FL, fibrous layer; R, ribosomes. Ribosome enumeration is shown in the supplementary data online, Fig. s1. (b–g) Complete serial sections of Cell 1 of MSB. (h) High magnification of (c). Arrows show cell surface membrane. Scale= 100 nm.

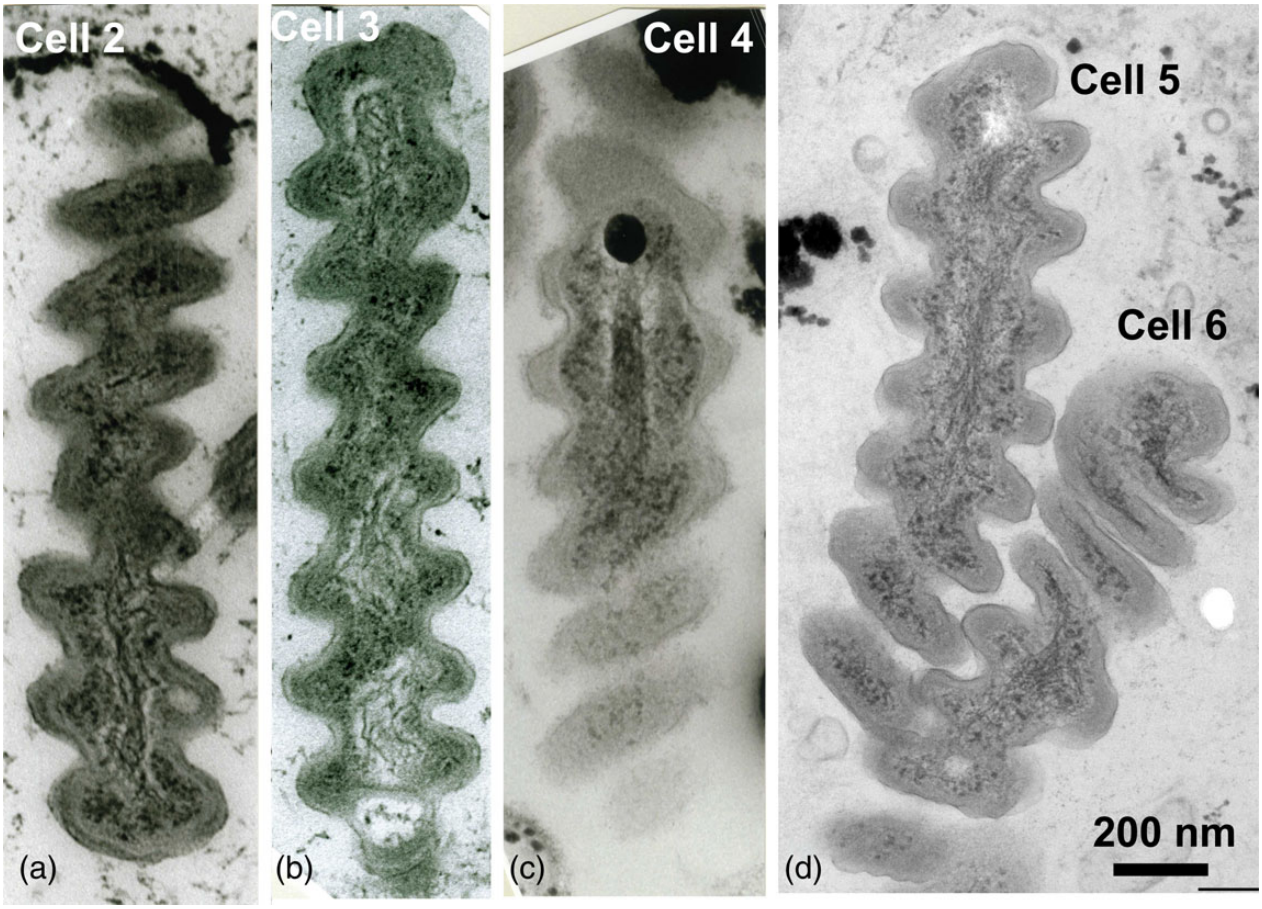


**Fig. 2.** Ultrathin sections of the central parts of Cell 2–6 of MSB. Complete serial sections of each cell are shown in the supplementary data online, Figs. s2–s5.

tomography at Nagoya (see the supplementary data online, Movies 3 and 4). The MSB were found to show either clockwise or counter-clockwise spiral (see the supplementary data online, Movies 3 and 4).

### Structome analysis of the MSB

Structome analysis of the MSB was performed by (i) examining complete serial sections of six cells (Figs. 1 and 2, and the supplementary data online, Figs. 1–5) and 3D movie files of 2.5 μm-thick sections (see the supplementary data online, Movies 3 and 4 and Table 1) and (ii) examining wide area of 1-μm-thick sections (see the supplementary data online, Fig. s6 and Table 2).

Table 1 shows the results of structome analysis of 70 -nm-thick serial sections of Cell 1 to Cell 6 and by HVEM tomography of Cell 7 to Cell 9. The total cell body length was 1.768 ± 0.478 μm; the total cell body diameter was 0.445 ± 0.050 μm; the cell body diameter was 0.185 ± 0.023 μm; the cell body pitch was 0.241 ± 0.046 μm (see also Fig. 5); the volume of the cells was 0.175 ± 0.073 $\mu m^3$; they had 322 ± 119 ribosomes in a cell and the density of the ribosomes was 216 ± 120/0.1 $\mu m^3$.

Table 2 shows the results of structome analysis of 40 cells by 1 μm-thick sections. The total length of the cell body was 1.62 ± 0.36 μm, and the total diameter of the cell body was 0.48 ± 0.05 μm.

Structome data from serial sections of 70 nm-thick sections, HVEM tomography of 2.5 μm-thick sections and data obtained by low magnification photos of 1 μm-thick sections were well matched (Tables 1 and 2), confirming the accuracy of measurements by all methods employed. With these images and movie files, we are able to produce a real 3D image of the MSB by handicraft of a wire (Fig. 5).

## Discussion

### Unique cell structure of the MSB

The MSB was found to have a unique fibrous layer. The presence of this fibrous layer is uncommon among bacteria and one of the features of the SPB. The fibrous layer must be

rigid enough to keep the cell shape. The role of the fibrous layer and their composition would be interesting problems in future research.

Some of the spiral bacteria are known to have periplasmic flagella between the inner plasma membrane and outer membrane [15,16], and the flagella of the spiral bacteria play an essential role in their motility [17,18]. However, the MSB had no flagella and may not be able to move. The reason why the MSB forms into such a complex spiral shape and benefits of the shape may be other interesting questions.

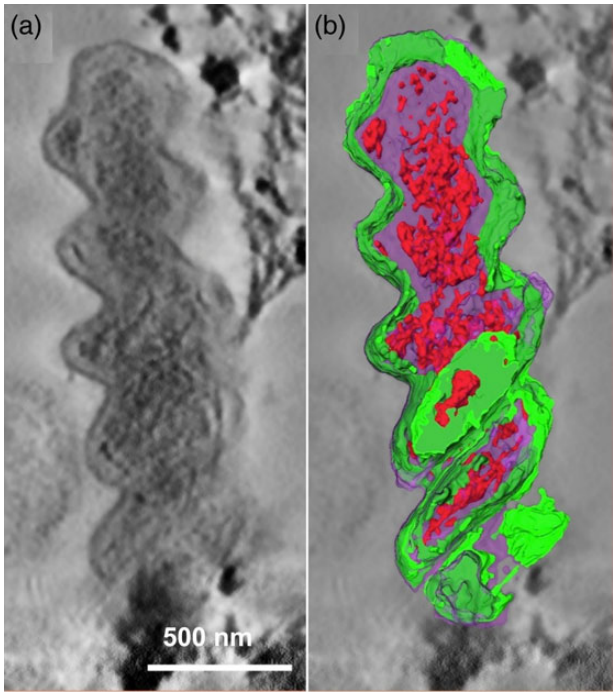


**Fig. 3.** 3D reconstructions of the MSB from 1-μm section. (a) Overall view. (b) Colored view. The fibrous layer is colored in green; the inner cytoplasm with a less electron-dense part is colored in purple; the more electron-dense part is colored red. Movie files are shown in the supplementary data online, Movies 1 and 2.

## Examination of the small MSB by HVEM tomography

It was not possible to infer the spiral shape from images of ultrathin sections of the MSB. It was also not possible to reconstruct good 3D images from serial ultrathin sections, because there were only six to eight sections for a whole cell. It was essential to obtain real 3D images of the MSB to observe and analyze them by HVEM tomography using thick sections that contained the whole MSB cells.

## Handedness of spiral bacteria

As stated above, the MSB showed either a clockwise or counter-clockwise spiral (see 'Results'). This indicates that either the same species could form spirals of both handedness, or the MSB comprises not one but at least two species, a question that awaits further study.

## Comparison of the structomes of MSB with those of *Escherichia coli* and *Mycobacterium tuberculosis*

Structome data of the MSB were compared with those of *E. coli* and *M. tuberculosis* (Table 3). The volume of the MSB was 19.7% of *E. coli* and 59.7% of *M. tuberculosis*. Thus, the MSB appears to be a very small organism compared with *E. coli* and *M. tuberculosis*.

*E. coli* grows rapidly and divides every 20 min. It had 25 770 ribosomes in a cell and showed density of 3640 ribosomes/0.1 $\mu m^3$ in average (Table 3). *M. tuberculosis* grows slowly and divides only every 20 h [19]. It had 1672

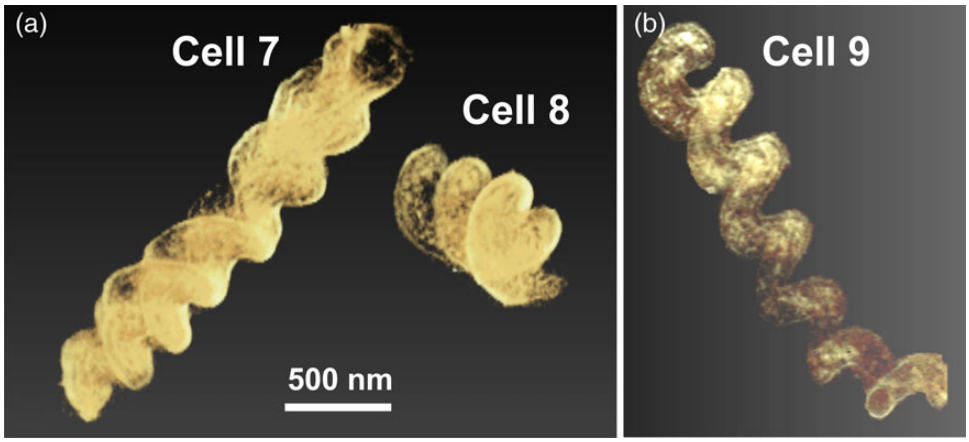


**Fig. 4.** 3D reconstructions of three MSB from 2.5-μm section. (a) Cell 7 and 8. By observing the supplementary data online, Movie 3, Cells 7 and 8 were found to have clockwise spiral. (b) Cell 9. By observing the supplementary data online, Movie 4, Cell 9 was found to have counter-clockwise spiral.

**Table 1.** Structome of the MSB using 70 nm-thick serial sections (Cells 1–6) and 3D movie files of 2.5 μm-thick sections (Cells 7–9)

| | Cell 1 | Cell 2 | Cell 3 | Cell 4 | Cell 5 | Cell 6 | Cell 7 | Cell 8 | Cell 9 | Average ± SD | Maximums | Minimums |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Total cell body length (μm) | 1.383 | 1.977 | 2.297 | 1.736 | 1.900 | 1.340 | 2.386 | 0.917 | 1.980 | 1.768 ± 0.478 | 2.386 | 0.917 |
| Total cell body diameter (μm) | 0.434 | 0.414 | 0.476 | 0.378 | 0.408 | 0.404 | 0.477 | 0.535 | 0.480 | 0.445 ± 0.050 | 0.535 | 0.378 |
| Cell body diameter (μm) | 0.191 | 0.188 | 0.209 | 0.181 | 0.169 | 0.150 | 0.200 | 0.220 | 0.160 | 0.185 ± 0.023 | 0.220 | 0.150 |
| Cell body pitch (μm) | 0.226 | 0.222 | 0.261 | 0.196 | 0.185 | 0.206 | 0.319 | 0.257 | 0.295 | 0.241 ± 0.046 | 0.319 | 0.185 |
| Cell volume ($\mu m^3$) | 0.121 | 0.217 | 0.290 | 0.119 | 0.201 | 0.102 | ND | ND | ND | 0.175 ± 0.073 | 0.290 | 0.102 |
| Number of ribosomes | 275 | 299 | 189 | 505 | 423 | 240 | ND | ND | ND | 322 ± 119 | 505 | 189 |
| Density of ribosomes (number/0.1 $\mu m^3$) | 227 | 138 | 65 | 423 | 210 | 235 | ND | ND | ND | 216 ± 120 | 423 | 65 |

ND, not determined.

**Table 2.** Structome of the MSB using 1 μm-thick sections

| | Number of cells measured | Average ± SD (μm) | Maximums (μm) | Minimums (μm) |
|---|---|---|---|---|
| Total cell body length | 40 | 1.62 ± 0.36 | 2.38 | 0.90 |
| Total cell body diameter | 40 | 0.48 ± 0.05 | 0.55 | 0.38 |

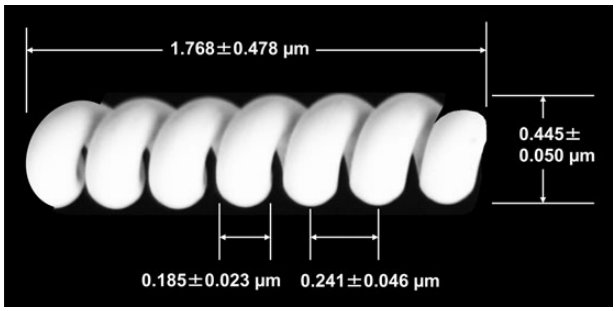


**Fig. 5.** A model of the MSB. Total cell body length is 1.768 ± 0.478 μm; total cell body diameter is 0.445 ± 0.050 μm; cell body diameter is 0.185 ± 0.023 μm and cell body pitch is 0.241 ± 0.046 μm.

ribosomes in a cell and showed density of 717 ribosomes/0.1 $\mu m^3$ in average (Table 3). It seems that a faster growth rate correlates with the presence of more ribosomes (and higher ribosome density).

The total number of ribosomes in the MSB was only 1.2% of that of *E. coli* and 19.3% of *M. tuberculosis*, and the density of the ribosomes was only 5.9% of that of *E. coli* and 30.1% of *M. tuberculosis*. If the number of ribosomes (and ribosome density) the reflect growth rate of organism, then the growth of the MSB must be very slow. Longer doubling time of the deep-sea microorganisms can be imagined because they live at low temperature (2–4°C) and in environments offering low nutrition. Structome analysis might be a useful method to estimate growth rate, when unknown microorganism cannot be cultured.

## Concluding remarks

We recognize animals and plants by looking at them. In other words, we identify their species mostly by external morphology. Morphological identification was also common even in the world of microorganisms, until DNA sequences were used for species identification.

We defined the structome as the quantitative and three-dimensional structural information of a whole cell at the electron microscopic level. It may be possible to identify species by structome analysis, if the microorganisms have unique external and internal structures. In fact, we proposed *Parakaryon myojinensis* by structome analysis as a new species that was found in a deep sea [1].

The deep sea occupies a vast volume of earth surface (70%), and yet little is known of the microorganisms that live there. We should start from knowing what kinds of microorganisms are living in the deep sea. Examining deep-sea specimens by electron microscopy may be one of the useful ways to find such microorganisms. Actually, we have found some strange-looking microorganisms in the deep sea (this study and Yamaguchi M *et al.*, unpublished). Such strange microorganisms might have very different cell structures, different cell functions or different metabolism from the currently known microorganisms. By studying such unusual microorganisms, we might be able to discover fundamental principles of life or evolution that are not known at present.

At present, it is difficult to culture most (99%) species of microorganisms [20]. To start investigation of uncultured microorganisms, structome analysis could be quite useful. More studies on structome analysis of uncultured microorganisms are expected to be carried out in not only deep sea but also in other fields such as intestinal microflora.

**Table 3.** Comparison of structomes between the MSB and other bacteria

| Species | Cell volume ($\mu m^3$, average) | Ribosome number in the cell (average) | Ribosome density (number/0.1 $\mu m^3$, average) | Reference |
|---|---|---|---|---|
| *E. coli* | 0.889 | 25 770 | 3640 | Yamada *et al.* (unpublished) |
| *M. tuberculosis* | 0.293 | 1672 | 717 | Yamada *et al.* (2015) [19] |
| MSB | 0.175 | 322 | 216 | Present study |
| Proportion to *E. coli* | 19.7% | 1.2% | 5.9% | |
| Proportion to *M. tuberculosis* | 59.7% | 19.3% | 30.1% | |

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## Conflict of interest

The authors have declared no competing interests exist.

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