Electron Microscopy and Structome Analysis of Unique Amorphous Bacteria from the Deep Sea in Japan

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Summary Structome analysis, quantitative and three-dimensional structural analysis of a whole cell at the electron microscopic level, is a useful tool for identification of unknown microorganisms that cannot be cultured. In 2012, we discovered a unique microorganism with a cell structure intermediate between those of prokaryotes and eukaryotes from the deep sea off the coast of Japan and named it Parakaryon myojinensis. We also reported another unique bacterium found in the same place that we named as Myojin spiral bacteria. Here, we report the third unique bacteria we discovered by structome analysis and 3D reconstruction using serial ultrathin sectioning of freeze-substituted specimens from the same place. The bacteria showed elongated flattened cell bodies with uneven surfaces. The cells consisted of outer amorphous materials, cell wall, cytoplasmic membrane, ribosomes, fibrous materials, and vacuoles. They had a total length of 1.82±0.40 µm, a total volume of 0.37±0.09 µm3, and had 1150±370 ribosomes within a cell; the density of the ribosomes in the cytoplasm was 312±41 per 0.1 fL. Each bacterium showed different shapes but appears to belong to a single species because they have similar size and volume, have similar internal structure, inhabit a confined area, and have similar ribosome density in the cytoplasm. We named it the ‘Myojin amorphous bacteria’ after the location of discovery and its morphology. This is the first report on the existence of amorphous bacteria.

Key words 3D reconstruction, Amorphous bacterium, Deep sea, Freeze-substitution fixation, Serial ultrathin sectioning, Structome.

In 2012, we discovered a unique microorganism that has cellular structures intermediate between prokaryotes and eukaryotes from the deep sea off the coast of Japan and named it P. myojinensis after the location of discovery and its intermediate morphology (Yamaguchi et al. 2012). 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 (Yamaguchi and Worman 2014, Yamaguchi 2015). We reported the second unique microorganism from the same place and named it Myojin spiral bacteria (MSB) (Yamaguchi et al. 2016).

Here, we report the third unique microorganism from the same place based on structome analysis [‘structome’ was defined as the ‘quantitative and three-dimensional structural information of a whole cell at electron microscopic level’ (Yamaguchi 2006, Yamaguchi et al. 2011a)] using serial ultrathin sectioning electron microscopy and 3D reconstruction of freeze-substituted specimens. This microorganism is unique since individuals of this species showed different shapes. We named it the ‘Myojin amorphous bacteria’ (MAB) after the location of discovery and morphology.

Materials and methods

Sample collection, specimen preparation, and elementary analysis

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 (Yamaguchi et al. 2012). 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 (Yamaguchi et al. 2011b, Yamaguchi 2013), and embedded in an epoxy resin. Serial ultrathin sections of 70-nm thickness were cut, picked up on slit grids (Yamaguchi et al. 2009, Yamaguchi and Chibana 2018), stained with uranyl acetate and lead citrate (Yamaguchi et al. 2005), and observed in a JEM-1400 electron mi-
Elementary analysis was undertaken on an ultrathin section after staining with uranyl acetate and lead citrate at 200 kV using the EDAX EDS system (EDAX Inc., Mahwah, NJ, U.S.A.) in a Tecnai 20 transmission electron microscope (FEI, Hillsboro, OR, U.S.A.).

**Structome analysis of the amorphous bacteria using serial sections**

Structome analysis of the amorphous bacteria using serial sections was undertaken on 10 individuals using micrographs of 15 to 35 complete serial sections for each individual. Image analyses were performed using Fiji (Image J, http://imagej.nih.gov/ij/) software (Kremer et al. 1996, Schindelin et al. 2012). Briefly, cell length was determined using the ‘Multi-point Tool’ in ImageJ/Fiji by tracing the cell wall using the polygonal selection menu in the ImageJ window and converting the area result above into \( \mu \text{m}^2 \) by multiplying the square of the ratio of scale (nm) on the image. The surface area \( (\mu \text{m}^2) \) of each cell was calculated as the cumulative area of a trapezium of the cell in each section using the formula for calculating the area of a trapezoid, where the perimeter of the cell walls in a given section and the previous section were used as the upper base and lower base, respectively, and the section thickness (70 nm) was used as the height. The volume \( (\mu \text{L}=\mu \text{m}^3) \) of each cell was calculated as the cumulative volume of cell segments having the cell’s cross-sectional area as the base and the section thickness (70 nm) as the height. Three-dimensional reconstruction of each cell was performed with TrakEM2 menu of ImageJ according to TrakEM2 tutorials (https://imagej.net/TrakEM2_tutorials).

Ribosomes, recognized as electron dense particles with 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 (Schindelin et al. 2012). The total number of ribosomes in each cell and the number of ribosomes per 0.1 \( \mu \text{L} \) of cytoplasm were calculated based on the volume of each cell determined as described above.

**Discussion**

**Preservation of cell structure in natural state**

It is essential to observe cell structures in a natural state at a high magnification to perform structome analysis of microorganisms. Since conventional chemical fixation often leads to the destruction of cell structures, rapid freeze-freeze substitution method should be employed for structome analysis (Yamaguchi et al. 2011b). Although samples from the deep sea must be fixed with glutaraldehyde aboard the ship (since rapid freezing cannot be performed aboard the ship), good preservation of cell structure can be obtained by employing rapid freezing for the samples fixed with glutaraldehyde (Yamaguchi et al. 2011b). Thus, the micrographs of MAB in the present study appear to show good ultrastructural preservation of cell structures appropriate for structome analysis.
Fig. 1. Low magnification view of an ultrathin section of the freeze-substituted MAB. There are 17 MAB in this field (arrows). The numbers indicate the cell number and the section numbers of the cell. For example, 1-11 shows the eleventh section of cell 1. Electron-dense inorganic materials (E) were found to contain iron (Fig. S7).

Fig. 2. High magnification of MAB. The bacterium consists of outer amorphous materials (A), outer membrane (OM), inner membrane (IM), ribosomes (R), fibrous materials (F), and vacuoles (V).
showing a three-layer structure. This double membrane structure is similar to the cell wall structure of Gram-negative bacteria (Madigan et al. 2015).

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

Structome data of the MAB were compared with those of *E. coli*, *M. tuberculosis*, and MSB (Tab. 2). The volume of the MAB was 32% of *E. coli*, 128% of *M. tuberculosis*, and 206% of MSB. Thus, the MAB appear to be a small organism compared to *E. coli*, but bigger than *M. tuberculosis* and MSB. *E. coli* grows rapidly and divides every 20 min. It has 26100 ribosomes in a cell with a density of 2840 ribosomes per 0.1fL on average (Table 2). *M. tuberculosis* grows slowly and divides only every 20 h (Yamada et al. 2015). It has 1670 ribosomes in a cell with a density of 717 ribosomes per 0.1fL on average (Table 2). The total number of ribosomes in the MAB was only 4.4% of *E. coli*, 68.9% of *M. tuberculosis*, and 357% of MSB. The density of the ribosomes was 11.0% of *E. coli*, 43.5% of *M. tuberculosis*, and 144% of MSB. Since the number of ribosomes (and ribosome

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**Table 1.** Structome of the MAB using 70-nm-thick serial sections.

<table>
<thead>
<tr>
<th>Cell</th>
<th>Number of sections</th>
<th>Cell length (µm)</th>
<th>Surface area (µm²)</th>
<th>Cell volume (µm³)</th>
<th>Total ribosome number</th>
<th>Ribosome density Number per 0.1 fL cytoplasm</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>15</td>
<td>1.05</td>
<td>2.13</td>
<td>0.21</td>
<td>500</td>
<td>236</td>
</tr>
<tr>
<td>2</td>
<td>23</td>
<td>1.61</td>
<td>4.04</td>
<td>0.44</td>
<td>1110</td>
<td>252</td>
</tr>
<tr>
<td>3</td>
<td>24</td>
<td>1.68</td>
<td>3.13</td>
<td>0.31</td>
<td>1050</td>
<td>338</td>
</tr>
<tr>
<td>4</td>
<td>24</td>
<td>1.68</td>
<td>3.28</td>
<td>0.34</td>
<td>1090</td>
<td>323</td>
</tr>
<tr>
<td>5</td>
<td>24</td>
<td>1.68</td>
<td>3.49</td>
<td>0.34</td>
<td>1000</td>
<td>295</td>
</tr>
<tr>
<td>6</td>
<td>24</td>
<td>1.68</td>
<td>3.49</td>
<td>0.34</td>
<td>1080</td>
<td>315</td>
</tr>
<tr>
<td>7</td>
<td>29</td>
<td>2.03</td>
<td>3.65</td>
<td>0.33</td>
<td>990</td>
<td>304</td>
</tr>
<tr>
<td>8</td>
<td>29</td>
<td>2.03</td>
<td>3.88</td>
<td>0.39</td>
<td>1340</td>
<td>345</td>
</tr>
<tr>
<td>9</td>
<td>33</td>
<td>2.31</td>
<td>2.50</td>
<td>0.55</td>
<td>1980</td>
<td>361</td>
</tr>
<tr>
<td>10</td>
<td>35</td>
<td>2.45</td>
<td>4.64</td>
<td>0.42</td>
<td>1360</td>
<td>348</td>
</tr>
<tr>
<td>Average</td>
<td>26</td>
<td>1.82</td>
<td>3.42</td>
<td>0.37</td>
<td>1150</td>
<td>312</td>
</tr>
<tr>
<td>SD</td>
<td>5.7</td>
<td>0.4</td>
<td>0.73</td>
<td>0.09</td>
<td>370</td>
<td>41</td>
</tr>
<tr>
<td>Minimum</td>
<td>15</td>
<td>1.05</td>
<td>2.13</td>
<td>0.21</td>
<td>500</td>
<td>236</td>
</tr>
<tr>
<td>Maximum</td>
<td>35</td>
<td>2.45</td>
<td>4.64</td>
<td>0.55</td>
<td>1980</td>
<td>361</td>
</tr>
</tbody>
</table>

**Fig. 3.** Complete serial sections of Cell 7. The numbers indicate the section number.
density) appears to reflect the growth rate of each organism (Yamada et al. 2017), the growth of the MAB may be slower than E. coli and M. tuberculosis. The longer doubling time of the deep-sea microorganisms can be expected because they live at low temperature (2–4°C) and in environments offering low nutrition.

Do the MAB belong to a single species?

In the world of organisms, individuals belonging to the same species exhibit the same shape. This is true not only in animals and plants but also microorganisms. In the present study, individual cells of MAB show different shapes. Do the MAB belong to a single species? We think they belong to a single species for the following reasons. 1) Individual cells of MAB show similar size and volume (Table 1). 2) They show similar internal cell structure (Figs. 1–3, S1–S6). 3) They inhabit a confined area. 4) They show similar ribosome density in the cytoplasm of the cell (this characteristic strongly suggests that they belong to the same species (Yamada et al. 2017). Genome analysis of the individual MAB cells observed in electron microscopy should be performed to confirm that they have the same genome and to establish their taxonomic position. Our team is now conducting research for establishing a method to analyze genomes of the individual microorganisms observed on ultrathin sections.

Do MAB change shapes?

If the MAB are flexible, they may change shapes with time like mycoplasma (Maniloff and Morowitz 1972). The mycoplasma has no cell walls and is enclosed by only a single cell membrane. On the other hand, the MAB have an external amorphous material, a cell wall, and a cytoplasmic membrane; similar cell surface structure to Gram-negative bacteria. Although we cannot determine if the MAB are rigid or flexible, we think they are rigid and does not change shape with time.

Division of MAB

Most rod-shaped bacteria proliferate by binary fission. They have similar size, shape, and structures along the cell body. However, the MAB have different sizes and shapes along the cell body because cross sections of each bacterium showed different shapes. How do the MAB proliferate? It may not be easy to control cell division under such situation. If the MAB are flexible, they may divide by fission like mycoplasma, showing coccoid shapes.

Table 2. Comparison of the structomes between the MAB and other bacteria.

<table>
<thead>
<tr>
<th>Species</th>
<th>Cell volume (µm³, average)</th>
<th>Ribosome number within the cell (Average)</th>
<th>Ribosome density (Number per 0.1fL, average)</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>E. coli</td>
<td>1.16</td>
<td>26100</td>
<td>2840</td>
<td>Yamada et al. (2017)</td>
</tr>
<tr>
<td>M. tuberculosis</td>
<td>0.29</td>
<td>1670</td>
<td>717</td>
<td>Yamada et al. (2015)</td>
</tr>
<tr>
<td>MSB</td>
<td>0.18</td>
<td>322</td>
<td>216</td>
<td>Yamaguchi et al. (2016)</td>
</tr>
<tr>
<td>MAB</td>
<td>0.37</td>
<td>1150</td>
<td>312</td>
<td>Present study</td>
</tr>
<tr>
<td>Proportion to E. coli</td>
<td>32%</td>
<td>4.4%</td>
<td>11.0%</td>
<td></td>
</tr>
<tr>
<td>Proportion to M. tuberculosis</td>
<td>128%</td>
<td>68.9%</td>
<td>43.5%</td>
<td></td>
</tr>
<tr>
<td>Proportion to MSB</td>
<td>206%</td>
<td>357%</td>
<td>144%</td>
<td></td>
</tr>
</tbody>
</table>

Fig. 4. 3D reconstructions of Cell 1 to Cell 10. a, front view; b, side view. Note each bacterium has a different shape.
cells connected by a membrane tubule (Maniloff and Morowitz 1972). However, we did not observe such images in our samples in the present study nor cell images that appear to be in cell division. The division mechanism of the MAB remains to be answered in the future.

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References


