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Prokaryote or eukaryote? A unique microorganism from the deep sea

Masashi Yamaguchi[1,*], Yuko Mori[2], Yoshimichi Kozuka[3], Hitoshi Okada[1,4], Katsuyuki Uematsu[5], Akihiro Tame[5], Hiromitsu Furukawa[2], Tadashi Maruyama[6], Cedric O'Driscoll Worman[7] and Koji Yokoyama[1]

[1]Medical Mycology Research Center, Chiba University, 1-8-1 Inohana, Chuo-ku, Chiba 260-8673, Japan, [2]System in Frontier Inc., 2-8-3 Shinsuzuharu Bldg. 4F, Akebono-cho, Tachikawa-shi, Tokyo 190-0012, Japan, [3]Vacuum Device Co. Ltd., 1285 Iijima-cho, Mito, Ibaragi 311-4155, Japan, [4]Integrated Imaging Research Support, Villa Royal Hirakawa 103, 1-7-5 Hirakawa-cho, Chiyoda-ku, Tokyo 102-0093, Japan, [5]Marine Works Japan, Ltd., 2-16-32 Kamariyahigashi, Kanazawa-ku, Yokohama 236-0042, Japan, [6]Marine Biodiversity Research Program, Japan Agency for Marine-Earth Science and Technology, 2-15 Yokosuka 237-0061, Japan and [7]Department of Biology, Francis Marion University, Florence, SC 29502, USA
*To whom correspondence should be addressed. E-mail: yama@faculty.chiba-u.jp

Abstract There are only two kinds of organisms on the Earth: prokaryotes and eukaryotes. Although eukaryotes are considered to have evolved from prokaryotes, there were no previously known intermediate forms between them. The differences in their cellular structures are so vast that the problem of how eukaryotes could have evolved from prokaryotes is one of the greatest enigmas in biology. Here, we report a unique organism with cellular structures appearing to have intermediate features between prokaryotes and eukaryotes, which was discovered in the deep sea off the coast of Japan using electron microscopy and structome analysis. The organism was 10 μm long and 3 μm in diameter, having >100 times the volume of *Escherichia coli*. It had a large 'nucleoid', consisting of naked DNA fibers, with a single nucleoid membrane and endosymbionts that resemble bacteria, but no mitochondria. Because this organism appears to be a life form distinct from both prokaryotes and eukaryotes but similar to eukaryotes, we named this unique microorganism the 'Myojin parakaryote' with the scientific name of *Parakaryon myojinensis* ('next to (eu)karyote from Myojin') after the discovery location and its intermediate morphology. The existence of this organism is an indication of a potential evolutionary path between prokaryotes and eukaryotes.

Keywords prokaryote, eukaryote, evolution, endosymbiotic theory, deep sea, freeze-substitution, structome

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Introduction

There are only two kinds of organisms on the Earth: eukaryotes, whose cells have a nucleus enclosed by a nuclear envelope, and prokaryotes, whose cells do not have such a nucleus [1,2]. Prokaryotic cells are typically a few micrometers in size, have simple cellular structures consisting of cytoplasm with a fibrous nucleoid, ribosomes, a plasma membrane and a cell wall. Eukaryotic cells have nearly 10,000 times the volume of prokaryotic cells, have a nucleus enclosed by a double membrane and show complex membranous cellular structures such as endoplasmic reticula, Golgi apparatuses, peroxisomes, lysosomes, endosomes and

various types of vacuoles. Additionally, they have either one or both of two distinct types of organelles viz. mitochondria and chloroplasts. Eukaryotic cells also have various types of cytoskeletal structures namely centrioles, microtubules and microfilaments [3].

Although eukaryotes are considered to have evolved from prokaryotes, there were no previously known examples of any intermediate forms between prokaryotic and eukaryotic organization [3,4]. In fact, the differences in cellular structure between prokaryotes and eukaryotes are so vast that the problem of how eukaryotes could have evolved from prokaryotes is one of the greatest enigmas in biology [5]. If eukaryotes had indeed evolved from prokaryotes, then there must have been viable organisms with intermediate cellular structures. The deep sea is one of the most likely environments to find such organisms because it exhibits the extreme environmental stability that allows the survival of morphologically stable organisms over long periods of time such as the coelacanth fish, which has been surviving with little morphological change for 400 million years in the deep sea.

There are two major hypotheses regarding the origin of eukaryotes [6,7]. According to the endosymbiotic theory, a moderately large, amoeboid, heterotrophic, anaerobic prokaryote engulfed aerobic bacteria, some of which were not digested but instead stabilized as endosymbionts, which became integrated into the host cell as mitochondria [8–11]. According to the autogenesis theory, the structures and functions of eukaryotic cells developed gradually from simple rudiments in prokaryotic cells [12,13]. There is still considerable debate about how eukaryotes originated [14,15].

We report here the existence of a deep-sea organism with morphology intermediate between those of prokaryotes and eukaryotes described using freeze-substitution electron microscopy and structome analysis. ('Structome' is defined as 'quantitative and three-dimensional structural information of a whole cell at the electron microscopic level' [16,17].) We then discuss its importance to the eukaryotic origin debate. We named the organism the 'Myojin parakaryote' with the scientific name of *Parakaryon myojinensis* ('next to (eu)karyote from Myojin') after the discovery location and its intermediate morphology, which places it neither with the prokaryotes nor with the eukaryotes.

Materials and methods

Samples were collected from hydrothermal vents at the Myojin Knoll (32°06.2N, 139°52.1E) off the coast of Japan at a depth of 1240 m in May 2010 [18]. All necessary permits were obtained for the described field studies from the Tokyo metropolitan government, which has jurisdiction over the field site. We collected small invertebrates, such as Polychaetes, and their associated microorganisms and fixed them with 2.5% glutaraldehyde. The samples were kept on ice and brought to the laboratory at Chiba University. They were snap-frozen, freeze-substituted [18] and embedded in an epoxy resin. Serial ultrathin sections were cut, picked up on slit grids [19] and observed in a JEM-1400 electron microscope (JEOL, Tokyo, Japan).

Structome analysis of *P. myojinensis* was undertaken using micrographs of 67 complete serial sections. Images of the cellular structure micrographs printed at a magnification of 20 000× were traced on transparency. Each image was digitized and stacked by the software Stack 'N' Viz (System in Frontier, Inc., Tokyo) to reconstruct the cell three dimensionally [17].

Results

Figure 1 shows an ultrathin section of *P. myojinensis*. This organism was found attached to the chaetae of a scale worm (Polynoide). It consisted of a cell wall, plasma membrane, large nucleoid, single nucleoid membrane and endosymbionts. By three-dimensional reconstruction from the complete serial sections of the cell (Fig. 2), *P. myojinensis* was found to be 10 µm long, with a diameter of 3 µm and a volume of 53 μm^3 (Table 1). There were three endosymbionts in the cell (Table 1, Fig. 2a and d). Endosymbiont 1 (E1) was very large and had a spiral shape (Fig. 2d). The four endosymbionts apparent in the sectioned image in Fig. 1 (labeled E) were found with three-dimensional reconstruction to be different parts of the one large spiral endosymbiont (E1) (Fig. 2a). The other two endosymbionts (E2 and E3; Fig. 2a and d) were both rod-shaped and small, together being only around one-tenth the volume of the large endosymbiont (Table 1).

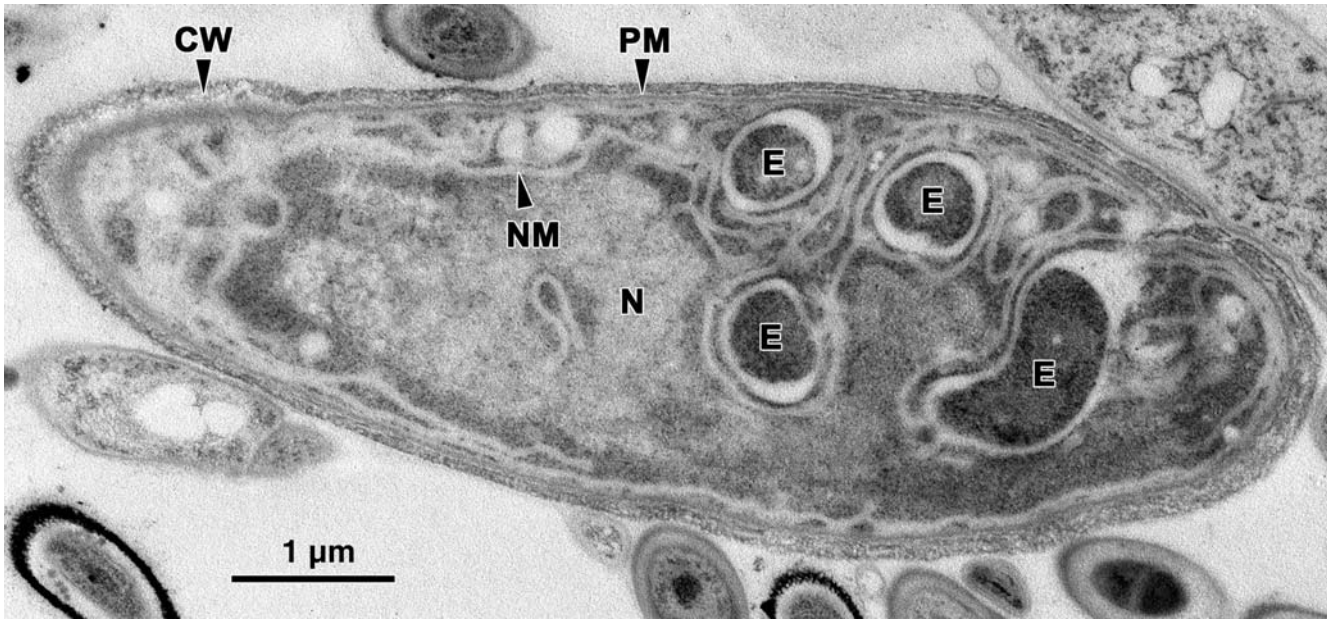

Fig. 1. An ultrathin section of *Parakaryon myojinensis*. Note the large irregular nucleoid (N) with single nucleoid membrane (NM), the presence of endosymbionts (E) and the absence of mitochondria. Also labeled are the cell wall (CW) and plasma membrane (PM).

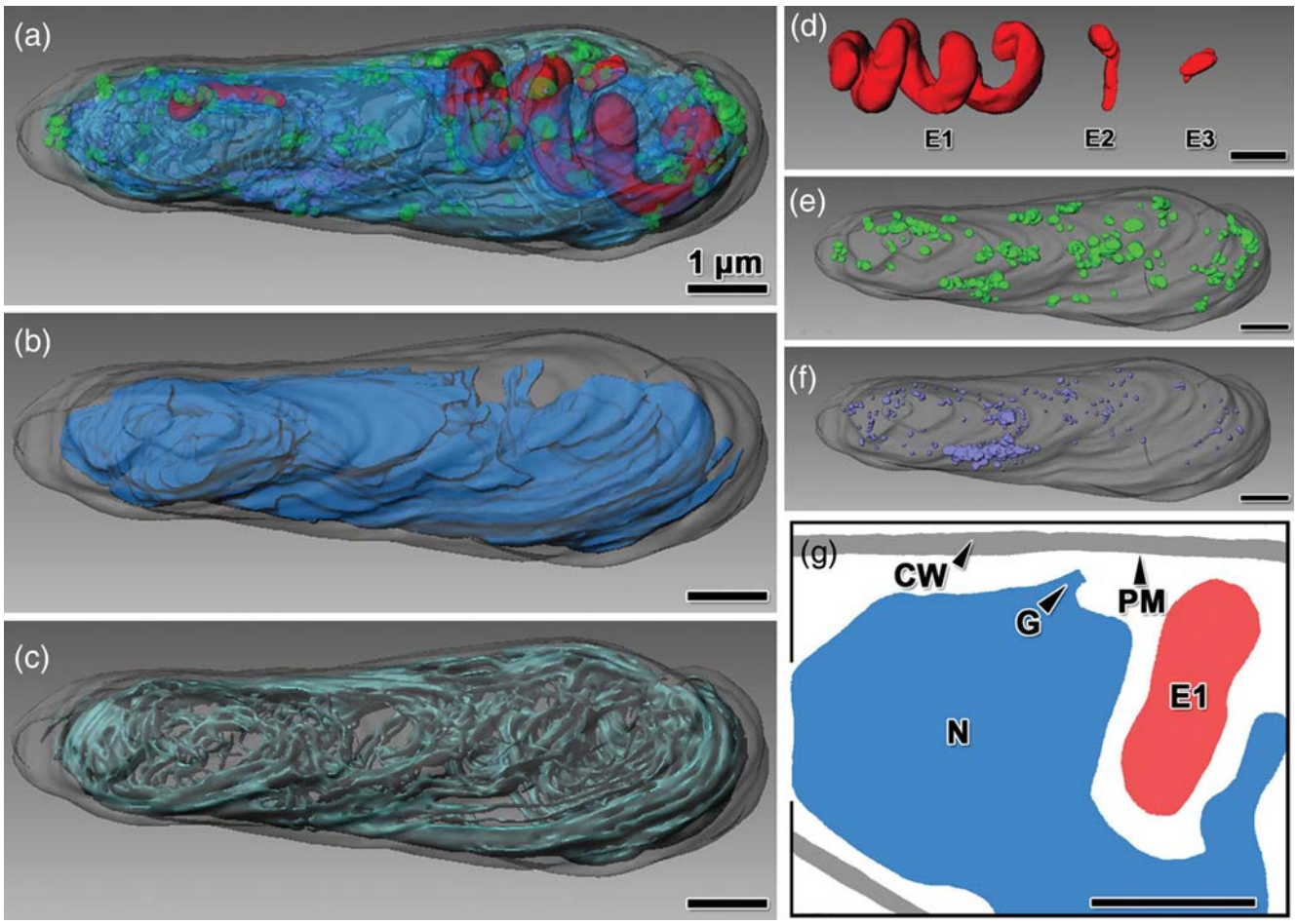

Fig. 2. The three-dimensional reconstruction of *P. myojinensis*. (a) The whole cell. (b) The 'nucleiod'. (c) The cytomembrane system of the host cell. (d) The endosymbionts. (e) The distribution of vacuoles in the host cell. (f) The distribution of the small granulated electron-transparent materials in the host cell. (g) Trace images of Fig. 3i showing how the nucleoid region (N) was defined by the inner most cytomembrane (nucleoid membrane) and smallest distance bridging the gaps (G). For 360° perspectives, see the supplementary data online.

Table 1. The numbers, sizes and volumes of all cell components in *P. myojinensis*

Cell/components	Number	Length × diameter	Volume (μm^3)		% total volume	
Cell	1	10.3 × 3.1 μm	52.6		100	
Cell wall	1		13.5		25.6	
Cytoplasm	1		39.1		74.4	
Nucleoid	1	9.0 × 2.1 μm	21.5		40.8	
Cytosol	1		9.1		17.2	
Plasma membrane	1		1.0	11.8	1.9	22.4
Cytomembranes	n. d.		1.7		3.3	
Ribosomes	n. d.		n. d.		n. d.	
Endosymbionts	3		2.6		4.9	
Endosymbiont 1		10.4 × 0.7 μm	2.3			
Endosymbiont 2		1.7 × 0.4 μm	0.2			
Endosymbiont 3		0.8 × 0.4 μm	0.1			
Phagosome space	3		2.3		4.3	
Vacuoles	102	236 nm (average diameter)	0.7		1.4	
Small granulated electron-transparent materials	150	155 nm (average diameter)	0.3		0.6	

n. d. = not determined.

At a higher magnification (Fig. 3), the interior cytoplasmic structures of the endosymbionts were found to be quite similar to those of modern eubacteria and consisted of fibrous nucleoids and ribosomes (Fig. 3a). However, the endosymbionts had no cell walls, but seemed to be only bounded by cell membranes (Fig. 3b).

The nucleoid of the host cell was not spherical but had an irregular shape (Figs. 1 and 2b), and occupied most of the host cytoplasm, accounting for 41% of the entire cell (Table 1, Fig. 4). It consisted of fibrous material and ribosomes (Fig. 3g). The fibrous material is likely composed of DNA fibers because the diameter of the fibers (2–3 nm) corresponds to that of DNA. Oddly, the 'nucleiod' was different from both the true nucleoids of prokaryotes and the true nuclei of eukaryotes in that it was enclosed by a single membrane, which we refer to as the nucleoid membrane (Figs. 1, 3g and i).

The cell had a complicated cytomembrane system (Figs. 2c and 3b), which occupied 1.7 times the area of the plasma membrane (Table 1) and formed the nucleoid membrane. The nucleoid membrane was not a closed membrane system, having gaps in places (Fig. 3i), and is different from the modern nuclear envelopes of eukaryotic cells that are made of closed double membranes.

The cell wall consisted of one layer, had a thickness of 80–120 nm (Figs. 1 and 3g) and occupied 26% of the cell volume (Table 1, Fig. 4). The plasma membrane appeared to be a typical three-leaflet structure of electron-dense, electron-transparent, and electron dense material (Fig. 3h) and had a thickness of 19.4 ± 3.9 nm ($n = 8$).

There were ~100 small vacuoles in the cell (Figs. 2e and 3e), which had an average diameter of 236 nm (Table 1) and occupied 1.4% of the cell volume (Table 1, Fig. 4). The cell also contained small granulated electron-transparent materials (Figs. 2f and 3f), which had an average diameter of 155 nm (Table 1) and occupied 0.6% of the cell volume (Table 1, Fig. 4). These structures are generally considered to be storage materials [20].

The cytosol, including the plasma membrane, cytomembranes and ribosomes, occupied 22% of the cell volume (Table 1, Fig. 4). There were no mitochondria, chloroplasts, a nucleolus, plastids, Golgi apparatuses, peroxisomes, centrioles, spindle pole bodies, nor microtubules in the cell.

Discussion

Features of *Parakaryon myojinensis*

A comparison of structomes was undertaken for *P. myojinensis*, *Saccharomyces cerevisiae* (a baker's yeast), *Exophiala dermatitidis* (a black yeast) and *Escherichia coli* (a colon bacillus) (Table 2). *Parakaryon myojinensis* is >100 times larger than *E. coli*, three times larger than *S. cerevisiae* and 1.5 times larger than *E. dermatitidis*

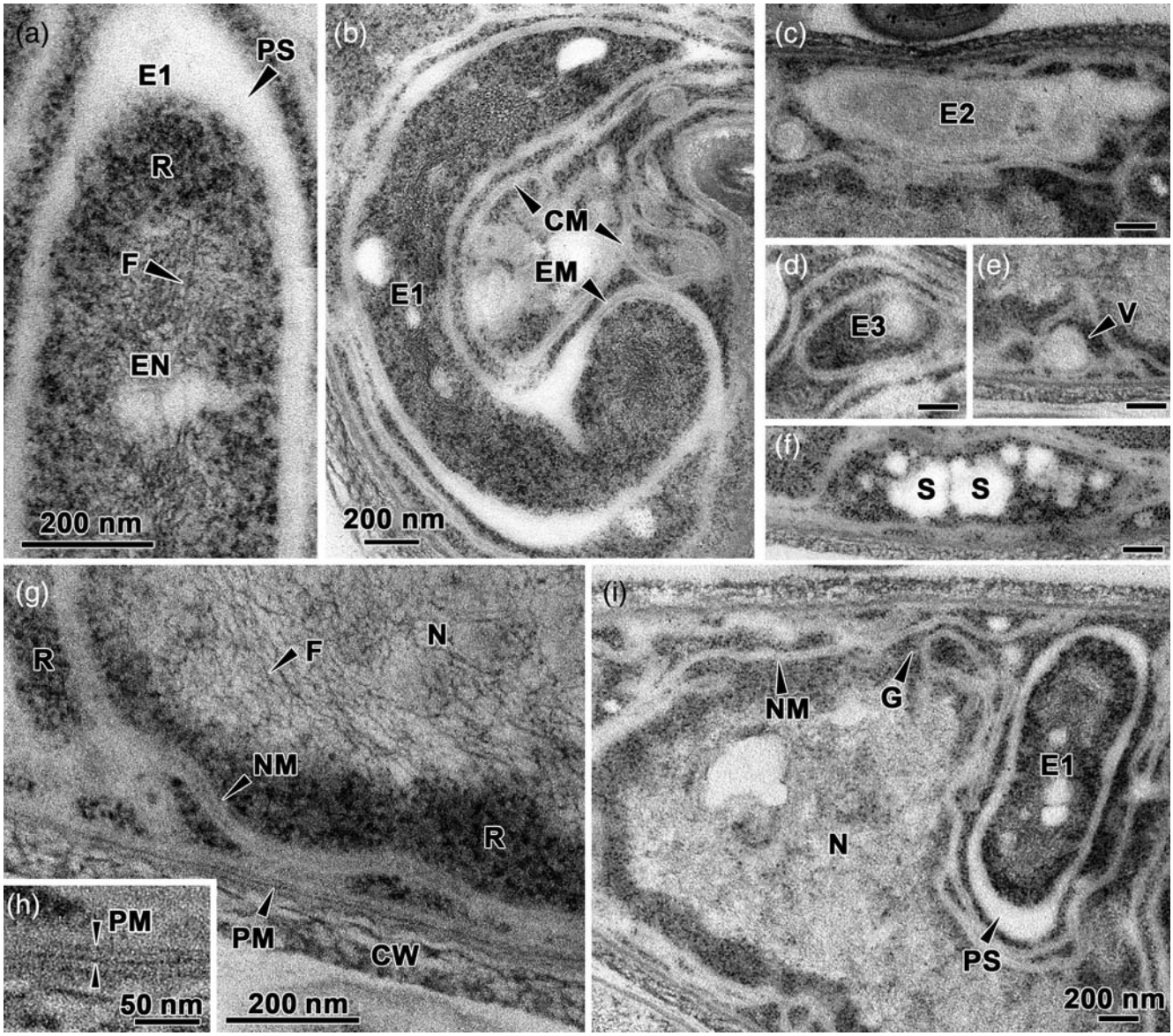

Fig. 3. *Parakaryon myojinensis* cellular components under high magnification. (a) and (b) The largest endosymbiont, Endosymbiont 1 (E1), showing the endosymbiont nucleoid (EN) with 2–3 nm DNA fibers (F), ribosomes (R) and the endosymbiont cell membrane (EM), as well as the cytomembranes (CM) and the phagosome space (PS) of the host. (c) The second largest endosymbiont (E2). (d) The smallest endosymbiont (E3). (e) A vacuole (V). (f) The small granular electron-transparent materials, which might be storage materials (S). (g) High magnification of the host nucleoid region (N) showing 2–3 nm DNA fibers (F), the nucleoid membrane (NM), ribosomes (R), the cell wall (CW) and the plasma membrane (PM). (h) High magnification of the plasma membrane (PM). (i) The nucleoid (N) enclosed by the nucleoid membrane (NM) with a gap (G). Also apparent is Endosymbiont 1 (E1) surrounded by phagosome space (PS). A traced image of (i) is shown in Fig. 2g. (c)–(f) scale bar, 200 nm. (a) and (g), (c)–(f) and (i) are of the same magnification.

(Table 2). The size of prokaryotes is typically confined to a few micrometers because their metabolism is dependent on the diffusion of molecules. Because *P. myojinensis* exceeds the normal size for prokaryotes, the organism likely has some kind of transport system within a cell. We do not know what kind of intracellular transport system this organism has or the function of the cytomembranes, and further study is necessary.

The most prominent feature of *P. myojinensis* is the large nucleoid, occupying >40% of the cell volume, whereas the nuclei of the yeasts occupy only 7–11% of the cell volume (Table 2). Endosymbionts occupy ~5% of the cell volume in *P. myojinensis*, which is bracketed by the mitochondrial volumes of *S. cerevisiae* (2%) and *E. dermatitidis* (10%) (Table 2). Further study is needed to clarify the function of the endosymbionts

and the nature of the symbiosis between the host and the endosymbionts. Table 3 summarizes the features of *P. myojinensis*.

When did *P. myojinensis* appear?

There are several examples of predatory or parasitic bacteria living within other prokaryote hosts [21,22]. These bacteria appear to be intact in the host cells: they show dense cytoplasms, keep their original rod shape and have cell walls. However, the cytoplasm of the host cells seems to be destroyed by the predatory or parasitic bacteria, as it becomes less dense and shows irregular morphology.

Is *P. myojinensis* made up of predatory bacteria and a host as in the above examples? If this is the case, then the specimen has a modern origin. We do not think so, however, because of the following three reasons.

First, in contrast to the above examples, *P. myojinensis* contained multiple endosymbionts of varying morphology (Fig. 2d). It is difficult to imagine that multiple bacteria of different species attacked a host at the same time. Also, it is unlikely that a very small bacterium lacking a cell wall (E3 in Fig. 2d) would be able to successfully attack a prokaryote that had a cell wall. Therefore, these endosymbionts cannot have been recently derived from independently living bacteria.

Secondly, because the cytoplasms of the host and the endosymbionts show orderly and electron-dense cellular structures, no digestion in either host or endosymbionts appears to have occurred. Hence, we believe that the host and endosymbionts are likely in good condition and able to coexist in a stable symbiosis.

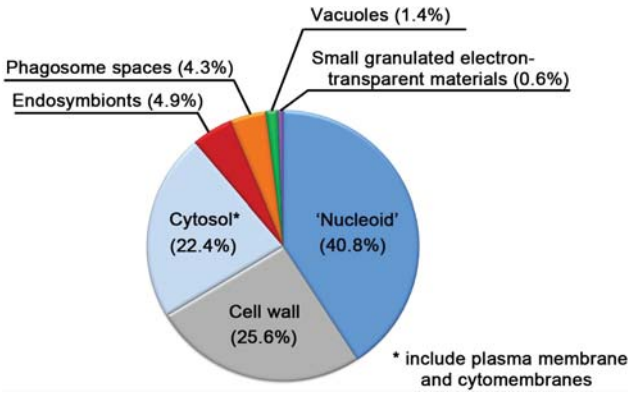

Fig. 4. The volumetric proportions of the cell components in *P. myojinensis*. This figure is available in black and white in print and in colour at *JEM* online.

Table 2. The structomes of *P. myojinensis*, *S. cerevisiae*, *E. dermatitidis*, and *E. coli*

	***Parakaryon myojinensis* (Present study)**	***Saccharomyces cerevisiae* [17]**	***Exophiala dermatitidis* [20]**	***Escherichia coli* [38]**
Cell size (length × diameter)	10.3 × 3.1 μm	3.9 × 3.2 μm	4.9 × 3.6 μm	not reported
Cell volume	52.6 μm^3	17.1 μm^3	36.0 μm^3	0.469 μm^3
Percent of whole cell volume				
Cell wall	25.6	17.0	21.8	not reported
Nucleoid or nucleus	40.8	10.5	7.3	
Endosynbiont or mitochondria	4.9	1.7	9.9	
Vacuoles	1.4	5.8	6.2	
Cytosol	22.4	64.0	47.5	
Other components	4.9	1.0	7.3	

Table 3. Features of *P. myojinensis*

1	Cell size	Much larger than ordinary prokaryotes; >100 times larger than *E. coli*; three times larger than *S. cerevisiae*
2	Nucleoid	Consists of prokaryote type DNA fibers and no nucleolus structure; very large and consists of >40% of the cell volume
3	Nucleoid membrane	Single membrane surrounding the nucleoid; pierced with gaps
4	Endosymbionts	Similar ultrastructure to modern eubacteria consisting DNA fibers and ribosomes; lack cell walls but enclosed by cell membranes
5	Other organelles	Cell wall, plasma membrane, complex cytomembrane systems, many vacuoles, small granular electron-transparent materials; none of the following: mitochondria, chloroplasts, plastids, Golgi apparatus, peroxisomes, centrioles, spindle pole body, microtubules

Lastly, if *P. myojinensis* originated due to a current interaction between predators and hosts, then there must be dense populations of predators and hosts, because predators need to find hosts quickly for survival once they are released from the previous host. For the past 12 years, we took >10 000 micrographs of microorganisms in the deep sea off the coast of Japan, but we have never before found a microorganism like *P. myojinensis*. This indicates that *P. myojinensis* lives at very low densities and is extremely unlikely to be a current interaction between predators and a host.

Parakaryon myojinensis is likely to be a stable symbiotic species that could have originated through an endosymbiotic event involving a relatively larger prokaryote and smaller bacteria as discussed by Margulis [9], and may even be a conservative descendent of the transitional lineage between prokaryotes and eukaryotes. The ancestor of *P. myojinensis* probably had no cell wall, enabling it to engulf bacteria. The phagocytosis machinery of the ancestor could have been well developed at the time the host engulfed the bacteria, considering that *P. myojinensis* itself has a complicated cytomembrane system and a phagosome-like organelle that contains endosymbionts. *Parakaryon myojinensis* most likely formed its cell wall at some point after the endosymbiotic process was complete. Also, although the endosymbionts in *P. myojinensis* have no cell walls, they likely had cell walls when they were engulfed by the host. The cell walls of the endosymbionts would then have been lost during a long-term symbiosis.

It may be possible to discover when *P. myojinensis* appeared and its phylogenetic position if fossil records are found or genetic work is carried out in the future.

Origin of mitochondria and nucleus

According to the endosymbiosis theory, the ancestor of mitochondria is believed to be a bacterium [23,24]. *Parakaryon myojinensis* had no mitochondria but instead had endosymbionts. The relationship must be a beneficial one for it to have lasted long enough for the endosymbionts to lose their cell walls and host to gain its cell wall. It seems likely that the endosymbionts in *P. myojinensis* are descendants of bacteria engulfed by a larger prokaryote in the past, thus the micrographs of the present study may provide evidence that the mitochondria in eukaryotes could indeed have evolved from internalized bacteria.

There are many hypotheses on the origin of eukaryote nucleus [25–28]. One hypothesis presumes that the eukaryote nucleus evolved gradually by the development of an inner cytomembrane system [13,29] while the others presume fusion or symbiosis between prokaryotes [30–32].

The nucleoid of *P. myojinensis* is not a true nucleus because it consists of DNA fibers rather than chromosomes, and is not enclosed by complete double nuclear membranes. Instead, the nucleoid is surrounded by a membrane composed of a single cytomembrane with occasional gaps. This nucleoid membrane could be a remnant form of a primitive nuclear membrane. If this is the case, then it follows that nuclear membranes could have evolved from the cytomembranes of prokaryotes by developing inner membrane systems. This line of reasoning contradicts the fusion or symbiosis theory of nucleus evolution.

There are debates about whether a nucleus was already formed when mitochondrial ancestors started their symbiosis with the host [33–35]. The nuclear region of *P. myojinensis* is not a completely formed eukaryotic nucleus but internalized endosymbionts are already in the host cell. This suggests that the nucleus was not necessarily formed when eubacteria started their endosymbiosis in the prokaryote host cell. Thus, the formation of the nucleus and transformation of bacteria into mitochondria might have proceeded independently.

The potential of electron-microscopic study of deep-sea microorganisms

There are many studies of new microorganisms that were isolated and cultured from the deep sea and characterized morphologically, genetically and/or biochemically [36]. However, culturing unknown microorganisms is always biased towards certain types of species with particular requirements for growth and reproduction. Considering that most microbes defy cultivation by standard methods [37], most organisms will be overlooked by these methods. Our strategy to find microorganisms morphologically intermediate between prokaryotes and

eukaryotes was by directly observing individual microorganisms collected from the deep sea. This strategy is time- and labor-intensive but has the advantage of sampling deep-sea microorganisms without bias toward organisms able to thrive in particular culturing conditions.

We also developed a method to observe the natural morphology of microorganisms at high resolution using freeze-substitution electron microscopy for examining the deep-sea samples [18]. We then used a serial ultrathin sectioning technique to conduct structome analysis [17,19], which enabled morphological identification of each microorganism. With these methods, we have observed and recorded a variety of microorganisms from the deep sea, many of which exhibit unusual morphologies. We think clues to important questions in evolution biology, such as the transition from prokaryote to eukaryote, the origins of mitochondria and nuclei, and even the origins of centrioles, spindle pole bodies, flagella and other organelles, could be obtained from observing deep-sea microorganisms with electron microscopy. In such a stable environment, there might be very little selective pressure for change in lineages originating 3.8 billion years ago when life first appeared on the Earth, leading to 'living fossils' still surviving only in the deep sea.

Concluding remarks

The existence of a prokaryote that has bacterial endosymbionts was not known previously. To our knowledge, this paper is the first to show such a unique microorganism. According to the endosymbiotic theory, a stable endosymbiosis between prokaryotes is the origin of primitive eukaryotes. This condition is what we appear to have observed by freeze-substitution electron microscopy in the deep-sea specimens. In addition to the endosymbionts, the microorganism had a nucleoid surrounded by a unique single membrane and complex membrane systems; thus, we named the microorganism 'parakaryote'. Of course, more specimens need to be collected and cultured to obtain the molecular data, including 16S rRNA genes, which will establish the evolutionary relationships between this microorganism and the prokaryotic and eukaryotic branches of life.

Supplementary data

Supplementary data are available at http://jmicro.oxfordjournals.org/.

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