Review

Deep-sea microorganisms and the origin of the eukaryotic cell

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SUMMARY

Living cells are deeply divided into two enormously divergent levels of complexity: prokaryotic and eukaryotic. Eukaryotes are thought to have developed from prokaryotic predecessors; however the large differences in their cellular structures results in equally large questions of how the process might have occurred. In 2012, in the deep-sea off the coast of Japan, we discovered a unique microorganism appearing to have cellular features intermediate between prokaryotes and eukaryotes. The organism, the Myojin parakaryote (tentatively named by Yamaguchi et al., 2012), was two orders of magnitude larger than a typical bacterium and had a large "nucleoid", consisting of naked DNA fibers, surrounded by a single layered "nucleoid membrane", and bacteria-like "endosymbionts", but it lacked mitochondria. This organism exemplifies a potential evolutionary path between prokaryotes and eukaryotes, and strongly supports the endosymbiotic theory for the origin of mitochondria and the karyogenetic hypothesis for the origin of the nucleus. In this review, we describe how the Myojin parakaryote was discovered, the features of this organism, the significance of the discovery, and perspectives on future research.

Key words: Prokaryotes, Parakaryotes, Mitochondria, Nucleus, Symbiosis, Electron microscopy, Freeze-substitution, Serial ultrathin sectioning, 3D reconstruction, Structome

INTRODUCTION

There are only two known ways of being a living cell: the prokaryotic and the eukaryotic. Prokaryotes include the Bacteria and Archaea. Prokaryotic cells are generally only a few micrometers in size, have simple cellular structures including cytoplasm with a fibrous nucleoid, ribosomes, a plasma membrane, and a cell wall. Eukaryotic cells are much more complex and include both single- and multi-cellular organisms; e.g., animals, plants, fungi, and protists. Both groups, particularly the eukaryotes, have wide cellular size ranges, however eukaryotic cells typically have

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nearly 10,000 times the volume of prokaryotic cells. Eukaryotic cells also have a nucleus enclosed by a double membrane and show complex membranous cellular structures: endoplasmic reticula, Golgi apparatuses, peroxisomes, lysosomes, endosomes, and various sizes and types of vacuoles. Additionally, eukaryotic cells have either one or both of two distinct types of organelles that contain their own DNA: mitochondria and chloroplasts. Eukaryotic cells also have various types of cytoskeletal structures: centrioles, microtubules and microfilaments (Fig. 1) (Stanier and van Niel, 1962; Mayr, 1998; de Duve, 1996).

Eukaryotes are thought to have evolved from prokaryotes, however, until recently, there were no known examples of intermediate forms between the vastly different prokaryotic and eukaryotic levels of organization (de Duve, 1996; Doolittle, 1998). In fact, the differences in cellular structure between prokaryotes and eukaryotes are so seemingly insurmountable that the problem of how eukaryotes could have evolved from prokaryotes is one of the greatest puzzles in biology (Koonin, 2010). One way to address this question is to find an organism with intermediate organization and examine its ultrastructure, DNA, and molecular machinery in detail. The deep-sea is one of the most likely environments to find such an organism because it exhibits the extreme environmental stability that allows for 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 (Dodson, 1979; Doolittle, 1980). In the endosymbiotic theory, a larger, amoeboid, heterotrophic, anaerobic prokaryote engulfed smaller aerobic prokaryotes, some of which stabilized as endosymbionts and became integrated into the host cell as mitochondria rather than be digested directly as a food source (Margulis, 1970; Whatley et al., 1979; Corsaro et al., 1999). In the autogenesis theory, the structures and functions of eukaryotic cells developed gradually from simple precursors in prokaryotic cells (Raff and Mahler, 1972; Nakamura and Hase, 1990). Significant debate about how eukaryotes originated continues in the present day (Kutschera and Niklas, 2005; Zimmer, 2009).

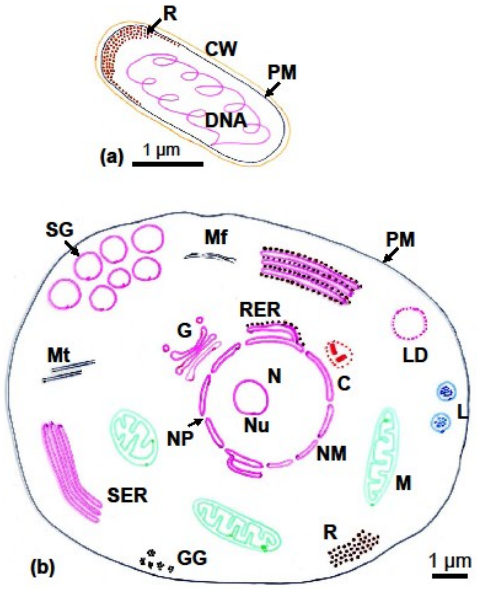

Fig. 1. Schematic diagrams of a prokaryotic cell (colon bacillus, a) and a eukaryotic cell (rat pancreas, b). C, centrioles; CW, cell wall; G, Golgi apparatus; GG, glycogen granules; L, lysosomes; LD, lipid droplets; M, mitochondria; Mf, microfilaments; Mt, microtubules; N, nucleus; NM, nuclear membrane; NP, nuclear pores; Nu, nucleolus; PM, plasma membrane; R, ribosomes; RER, rough endoplasmic reticulum; SER, smooth endoplasmic reticulum; SG, secretion granules (From Yamaguchi (2013) with permission).

In 2012, in the deep-sea off the coast of Japan, we found a unique microorganism which appears to have an intermediate cellular structure between those of prokaryotes and eukaryotes (Yamaguchi et al., 2012). This organism was described using freeze-substitution electron microscopy and structome analysis (*Structome* is defined as the "quantitative and three-dimensional structural information of a whole cell at the electron microscopic level" (Yamaguchi, 2006;

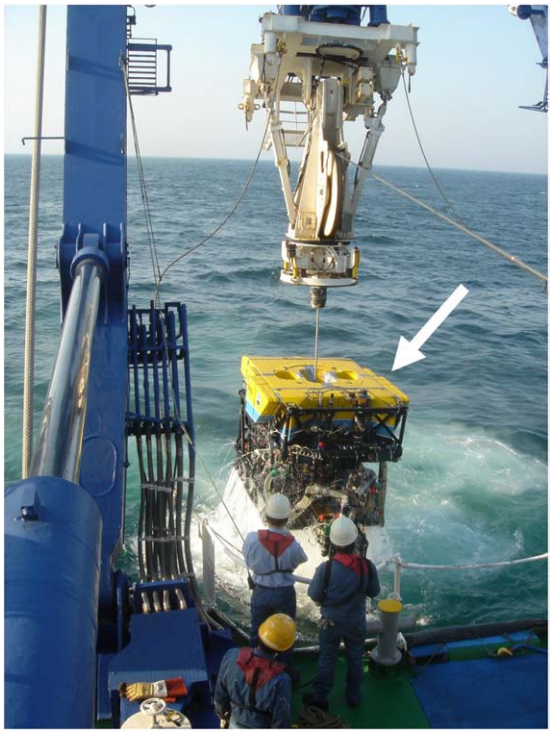

Fig. 2. The remotely operated vehicle, Hyper-Dolphin (arrow).

Yamaguchi et al., 2011a)). The organism was named the "Myojin parakaryote" with the scientific name of *Parakaryon myojinensis* ("next to (eu)karyote from Myojin") after the discovery location and its intermediate morphology between the prokaryotes and the eukaryotes (Yamaguchi et al., 2012). In this review, we describe how the Myojin parakaryote was discovered, the features of this organism, the significance of the discovery, and perspectives on future research.

SAMPLE COLLECTION

In May 2010, we left Yokosuka harbor on the research vessel *Natsushima* heading to the Myojin Knoll (32°08.0'N, 139°51.0'E), which is located about 100 km south of Hachijo Island off the coast of Japan. Samples were collected from hydrothermal vents at a depth of 1,240 m using a remotely operated vehicle, Hyper-Dolphin (Fig. 2). There was dense population of larger creatures

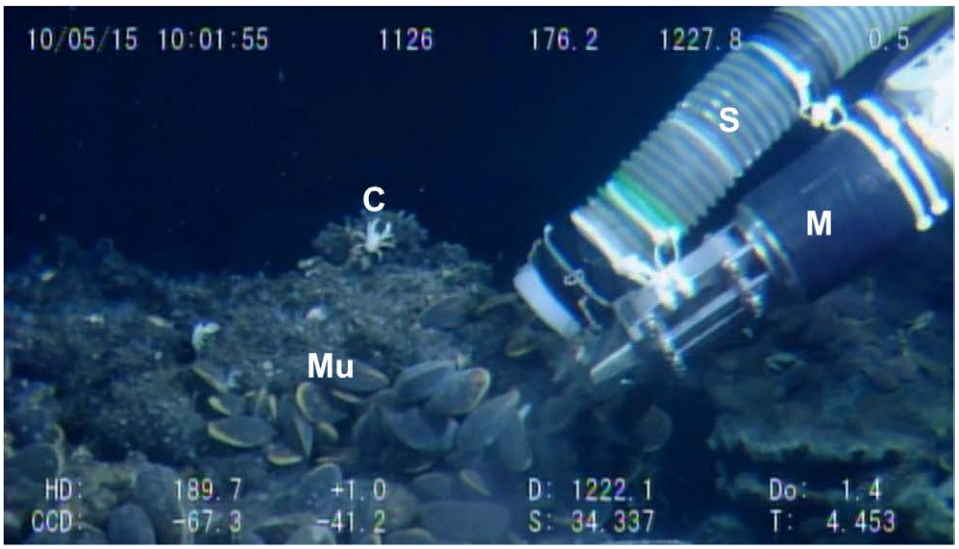

Fig. 3. The rich community of deep-sea organisms at a depth of 1,227.8 m on Myojin Knoll; including mussels (Mu) and crabs (C). Samples were collected with a slurp gun (suction sampler, S) or with a manipulator (M). Sample collection lasted 2 hours for each dive and was recorded on video. This picture was taken on the 15th of May, 2010. Other information on time (10:01:55AM), dive number (1,126), direction (176.2), height from the sea bottom (0.5 m), type of main camera (HD), sub-camera (CCD), depth of the machine (1,222.1 m), salinity (34.337 PSU), dissolved oxygen concentration (1.4 mL/L), and temperature (4.453°C) was recorded. *P. myojinensis* was collected in this dive.

Fig. 4. A scale worm (Polynoidae) that we collected from deep sea and its many chaetae.

(e.g., crabs and mussels) near the hydrothermal vents (Fig. 3). We collected small invertebrates, such as Polychaetes, and their associated microorganisms. Most of the collected creatures were alive when they were lifted onto the deck of the ship. The animals were photographed directly or under a stereomicroscope to record whole morphological features and aid in species identification (Fig. 4). The specimens were fixed with 2.5% glutaraldehyde in sea water, kept on ice, and transported to the laboratory in Chiba University.

ELECTRON MICROSCOPIC OBSERVATION OF MICROORGANISMS BY CONVENTIONAL CHEMICAL FIXATION

The glutaraldehyde-fixed samples were post-fixed with 1% osmium tetroxide, dehydrated in a graded series of ethanol, and embedded in epoxy resin. Ultrathin sections were cut to a thickness of 70 nm, stained with uranyl acetate and lead citrate, covered with Super Support Film (Nisshin EM, Tokyo, Japan), and observed in a JEM-1400 electron microscope (JEOL, Tokyo, Japan) at 100 kV.

Many microorganisms were found associated with the chaetae of scale worms (Polynoidae) (Fig. 5). Although the ultrastructure of microorganisms cannot be observed by light microscopy, they were

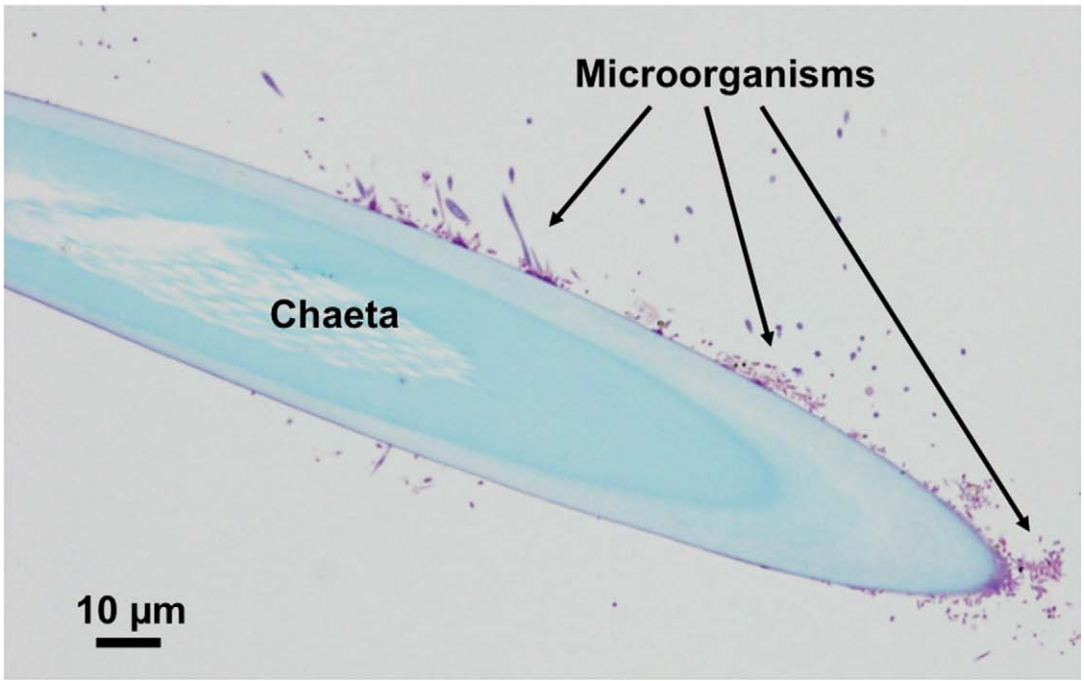

Fig. 5. A transverse section of a scale worm chaeta and the many associated microorganisms on the surface of the chaeta.

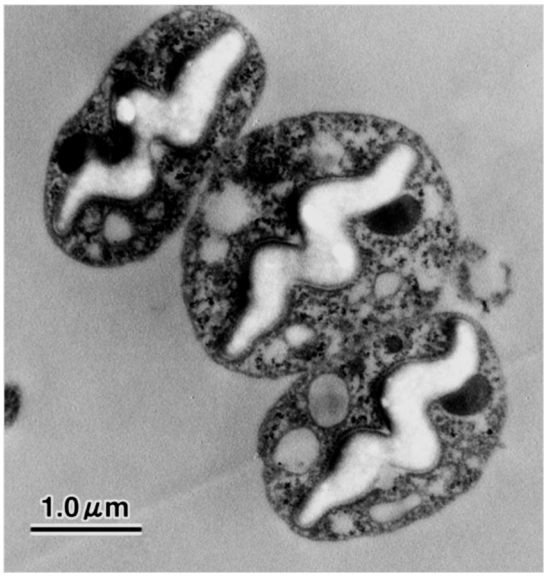

Fig. 6. An example of unusual deep-sea microorganisms from Myojin Knoll with an internal cell wall-like gull-shaped structure. There were 4 individuals side by side (only 3 are shown here), suggesting division by binary fission.

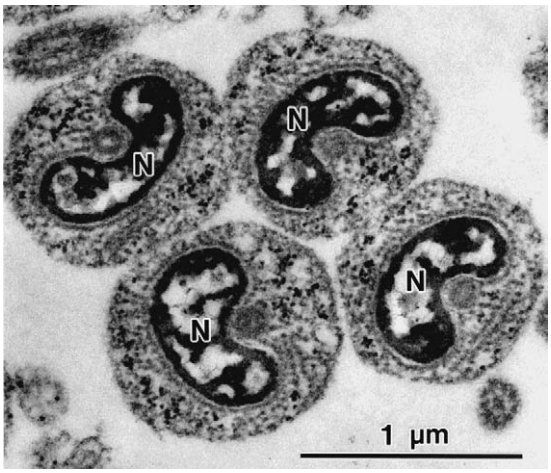

Fig. 7. An example of unusual deep-sea microorganisms from Myojin Knoll with unique comma-shaped nuclei-like structure (N). They seem to have no mitochondria.

clearly observed by electron microscopy. Fig. 6 shows spherical microorganisms with cell wall-like structures inside the cells. They measure about 2.5 μm in diameter and are postulated to divide by

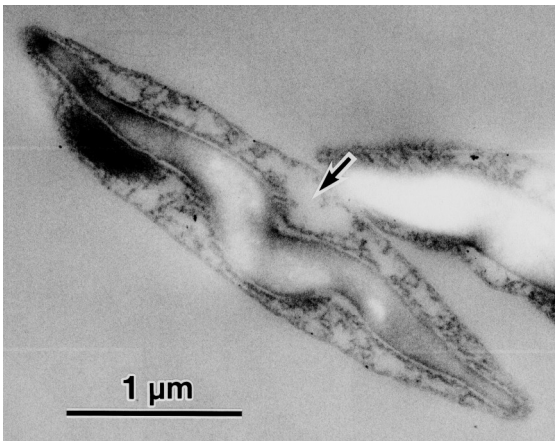

Fig. 8. An example of unusual lens-shaped deep-sea microorganisms with internal cell wall-like gull-shaped structures. Note the structure of the cytoplasm was heavily damaged by the conventional specimen preparation (arrow).

binary fission because there were 4 individuals side by side. If the electron-lucent gull-shaped structures are indeed internal cell walls, these bacteria are the first observed with such a unique morphology. Cell walls on known bacterial cells are always external to the cell membrane, thus this configuration must have divergent and unknown advantages. This inner cell wall structure might be an example of the diverse and seemingly bizarre adaptations that are found in extreme environments, such as the deep sea, but are currently unknown on the surface of the Earth.

Fig. 7 shows another example of the poorly-studied microbe community in the deep sea. These spherical microorganisms measure about 1.0 μm in diameter. Like the organisms in Fig. 6, they may divide by binary fission since there are 4 individuals side by side. These microbes have unique comma-shaped nuclei-like structure and lack mitochondria. Fig. 8 shows yet another example of the types of interesting life forms we are finding in the deep sea. In this case, the microorganism is lens-shaped with a gull-shaped cell wall-like structure inside the cell very similar to that seen in Fig. 6.

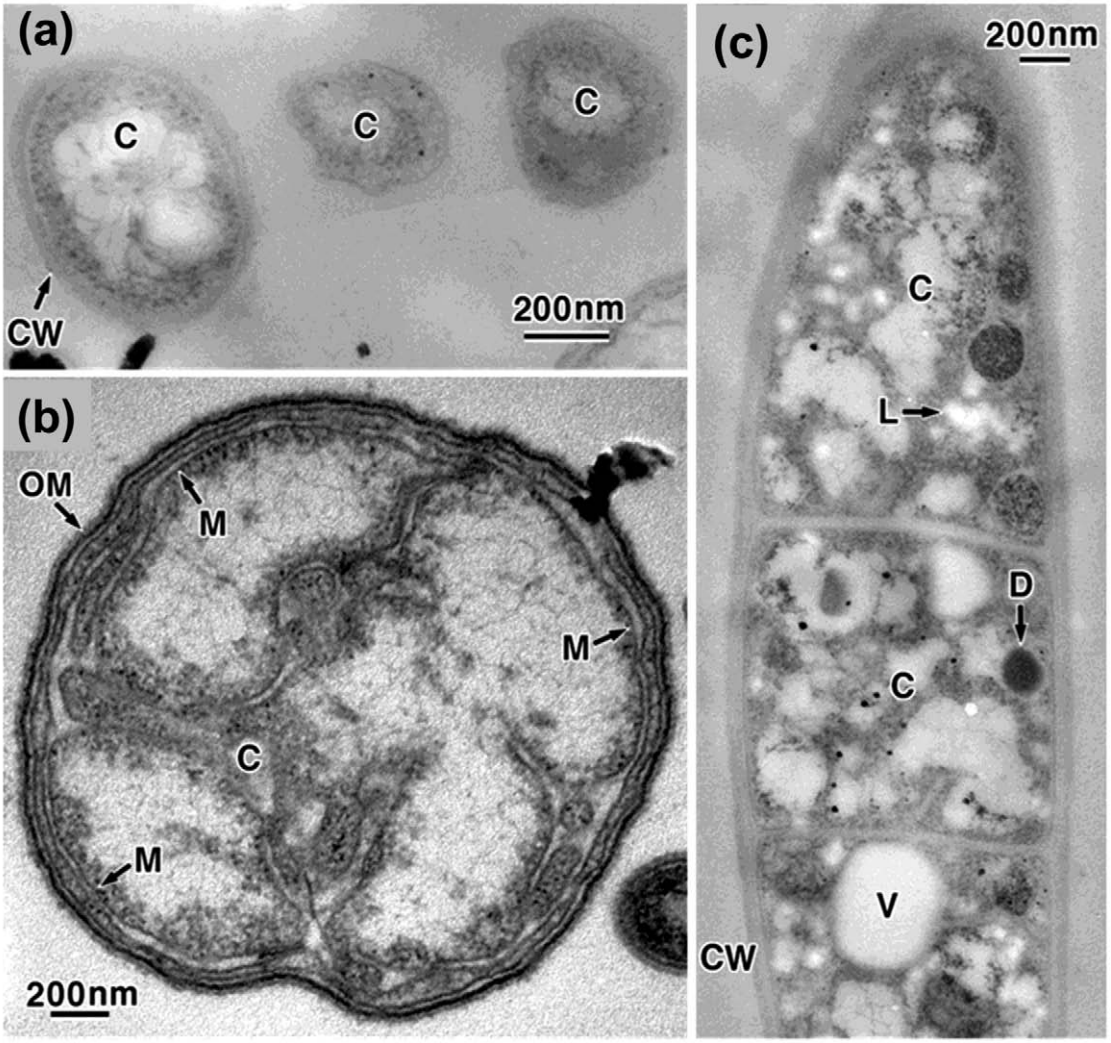

Fig. 9. Ultrathin sections of deep-sea microorganisms prepared using conventional chemical fixation (CF method). Samples were fixed with 2.5% glutaraldehyde aboard the ship, transferred to the laboratory, post-fixed with 1% osmium tetroxide, and embedded in epoxy resin. Note that membranes are not smooth (OM in b) and cytoplasmic structures appear to be distorted and extracted (a–c). C, cytoplasm; CW, cell wall; D, electron-dense components; L, electron-lucent components; M, membrane; OM, outer membrane; V, vacuole (also for Fig. 10) (From Yamaguchi et al. (2011b) with permission).

Through the initial observations shown in Figs. 6–8, we noticed that it is necessary to observe deep-sea microorganisms in three-dimensions to truly understand the whole cell structure. We also noticed that the ultrastructure of microorganisms is heavily damaged by conventional chemical fixation and thus there was a need to develop methods for observing intact morphology at high resolutions (Therefore, the above descriptions, which are based only on one ultrathin section of chemically fixed cells, must be considered preliminary until we examine the 3D ultrastructure of the cells with the more precise methods elucidated below).

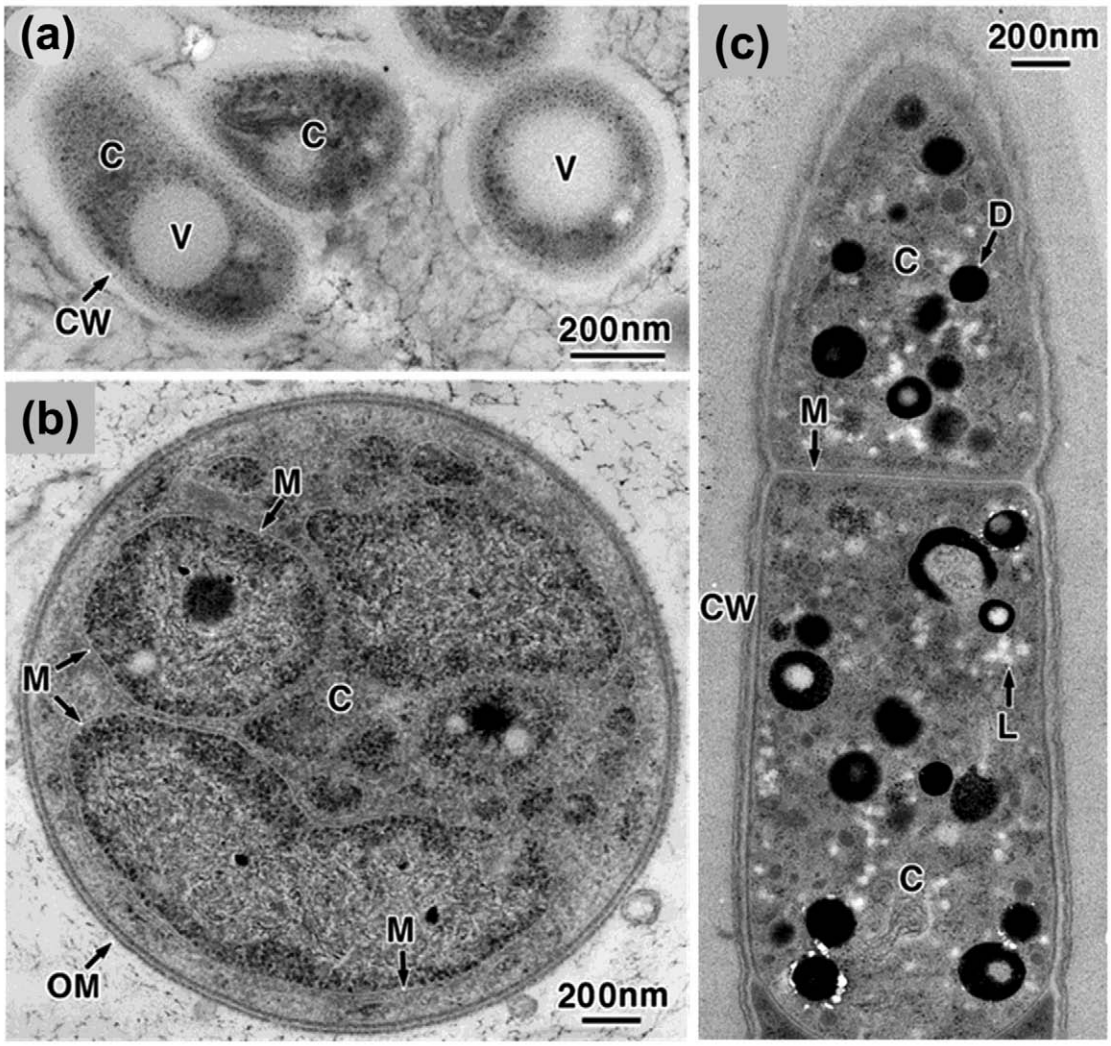

Fig. 10. Ultrathin sections of deep-sea microorganisms prepared using freeze-substitution after glutaraldehyde fixation (CF-FS method). Samples were fixed with 2.5% glutaraldehyde aboard the ship, transferred to the laboratory, cryofixed with melting propane, freeze-substituted in acetone containing 2% osmium tetroxide at −80°C, and embedded in epoxy resin. Ultrathin sections were cut to a thickness of 70 nm, stained with uranyl acetate and lead citrate, covered with Super Support Film, and examined in an electron microscope. Note that membranes are very smooth (OM in b, M in c), cytoplasm is filled with electron-dense components and vacuoles (a–c), and the cell (b) and vacuoles (a) are nearly spherical, showing natural forms and high-resolution images of ultrastructures in deep-sea microorganisms (From Yamaguchi et al., (2011b) with permission).

DEVELOPMENT OF BETTER SPECIMEN PREPARATION METHODS

We knew that rapid-freeze freeze-substitution fixation would preserve the natural ultrastructure of our deep-sea microorganisms at high resolution. However, the method cannot be performed on a research vessel, because the time for specimen preparation on board is limited and refrigerants such as propane are too dangerous to bring into the vessel's laboratory. We thought rapid-freeze freeze-substitution even after glutar-

aldehyde fixation would give a significantly better preservation of the ultrastructure of deep-sea specimens because we previously had good results from freeze-substitution of glutaraldehyde-fixed yeast cells (Yamaguchi et al., 2005).

The chaetae with associated microorganisms were cut from glutaraldehyde-fixed scale worms, rinsed with phosphate buffer (pH 7.2), and sandwiched between two copper discs. They were snap -frozen by being plunged into melting propane kept in liquid nitrogen. The specimens were freeze -substituted in acetone containing 2% osmium tetroxide at –80°C for 2 to 6 days and embedded in epoxy resin (Yamaguchi et al., 2011b) (We refer to this new method as CF-FS (chemical fixation-freeze-substitution) method, as opposed the conventional chemical fixation method (CF method)). Ultrathin sections were stained with uranyl acetate and lead citrate, covered with Super Support Film, and observed in a JEM-1400 electron microscope the same as with the conventional method.

Fig. 9a shows bacteria-like microorganisms, 0.3 to 0.5 μm diameter, prepared using CF method. They appear to consist of a cell wall/outer cell membrane and cytoplasm. Parts of the cytoplasm in the center are often devoid of electron-dense components, have fine filaments, and seem to have had the contents artificially extracted. Fig. 10a shows bacteria-like microorganisms similar to those in Fig. 9a, but prepared using CF-FS method. They also appear to consist of a cell wall/outer cell membrane and cytoplasm. The cytoplasm appears to contain electron-dense components and an electron-lucent vacuole, which is spherical in shape. CF-FS method preserves the natural morphology of the cell, including the vacuole, while CF method disrupts fragile cellular structures and only allows visualization of robust structures.

Fig. 9b shows a 2.2 to 2.5 μm diameter microorganism prepared using CF method. It appears to consist of an outer membrane and cytoplasm with several cellular compartments that are surrounded by membranes but appear to be devoid of electron-dense materials inside, instead showing scattered electron-opaque materials. Fig. 10b shows a microorganism similar to the one in Fig.

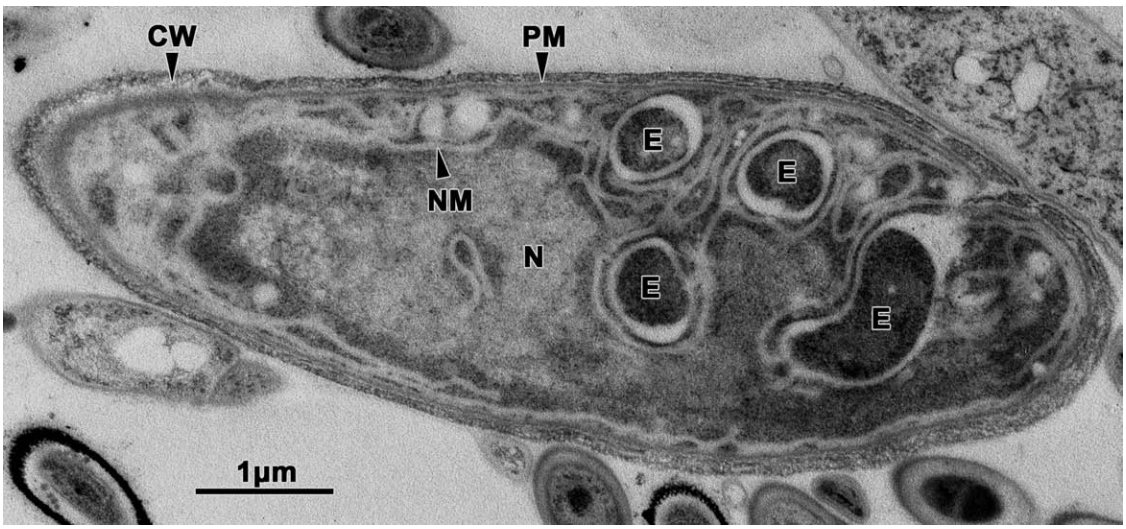

Fig. 11. An ultrathin section of *Parakaryon myojinensis*. Note the large irregular "nucleoid" (N) with single layer "nucleoid membrane" (NM), the presence of endosymbionts (E), and the absence of mitochondria. Also labeled are the cell wall (CW) and plasma membrane (PM) (From Yamaguchi et al. (2012) with permission).

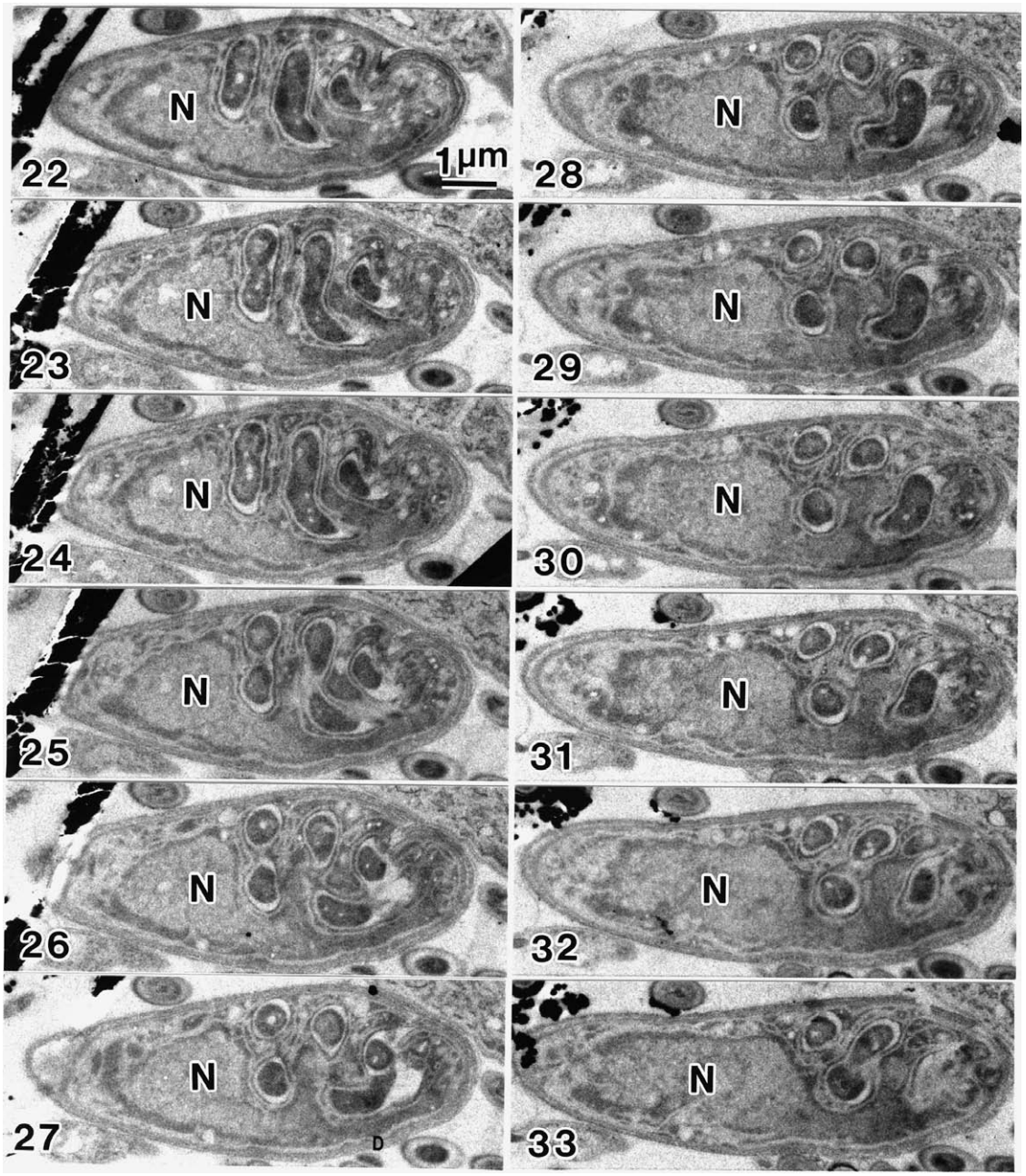

Fig. 12. Serial sections of *P. myojinensis*. The numbers at the lower left are sequential from the first section. 12 out of 67 sections. N, nucleoid.

9b, but prepared using CF-FS method. It appears to consist of an outer membrane and a cytoplasm which has several compartments surrounded by membranes. The outer membrane has a very smooth circumference consisting of an outer electron-dense leaflet, a middle electron-lucent leaflet, and an inner electron-dense leaflet. Thus, CF-FS method prevents extraction of cytoplasm

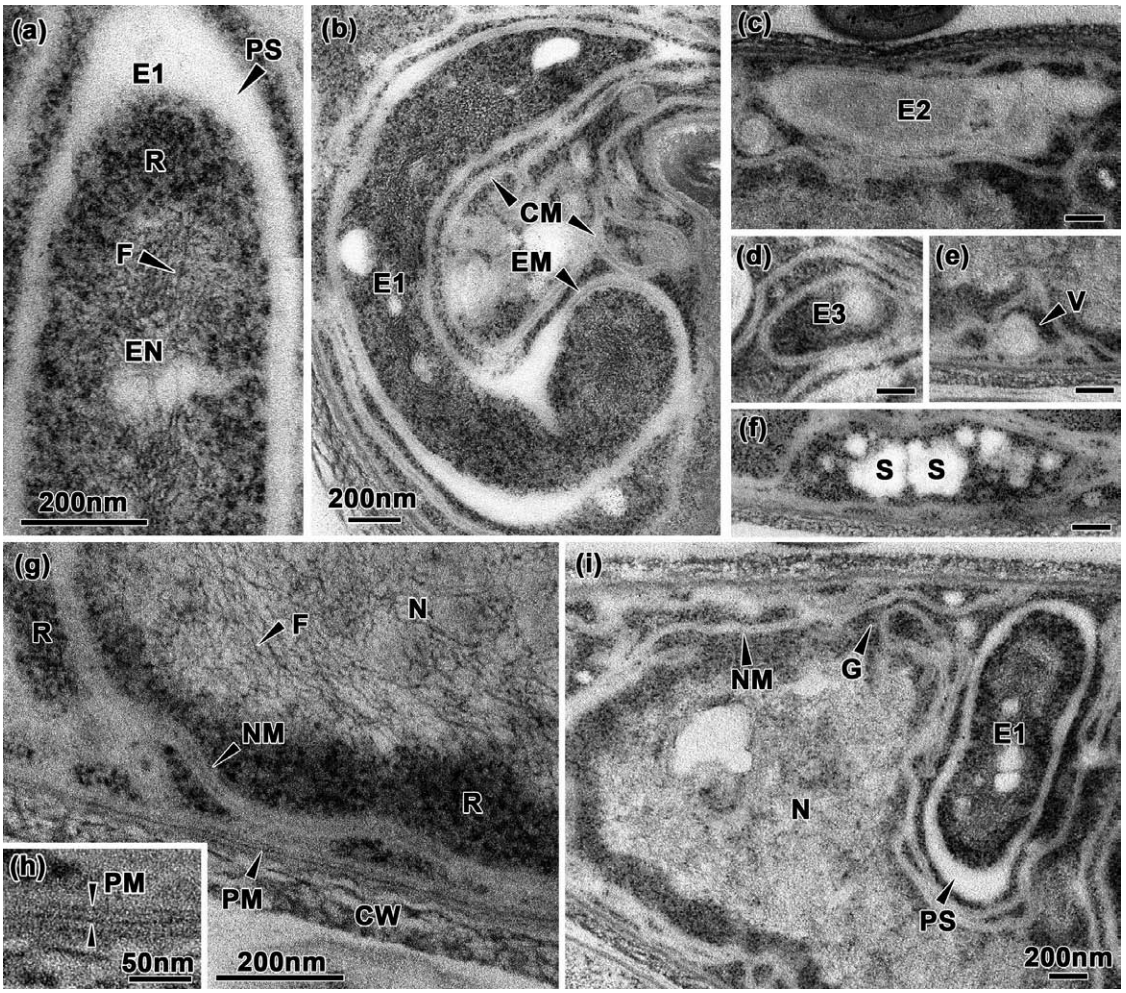

Fig. 13. The cellular components of *P. myojinensis* under high magnification. (a) and (b) The largest endosymbiont, Endosymbiont 1 (E1), showing the endosymbiont nucleoid (EN) with DNA fibers (F), ribosomes (R), and the endosymbiont cell membrane (EM), as well as the cytomembranes (CM) and the phagosome space (PS) of the host. (c) The second largest endosymbiont (E2). (d) The smallest endosymbiont (E3). (e) A vacuole (V). (f) Small granular electron-transparent materials, which might be storage materials (S). (g) High magnification of the host "nucleoid" region (N) showing 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. 14g (From Yamaguchi et al. (2012) with permission).

components and preserves natural cell morphology.

Fig. 9c shows an apical part of a filamentous multicellular microorganism with cell walls prepared using CF method. The cytoplasm of each cell contains several components, some of which are electron-dense while others are electron-lucent. Fig. 10c shows an apical part of a similar filamentous multicellular microorganism prepared using CF-FS method. The cytoplasm of each cell is filled with similar components in both specimens, however the morphology of each component appears clearly only in the CF-FS method specimen.

As these examples show, by applying rapid-freeze freeze-substitution after glutaraldehyde fixation (CF-FS method), it is possible to observe the

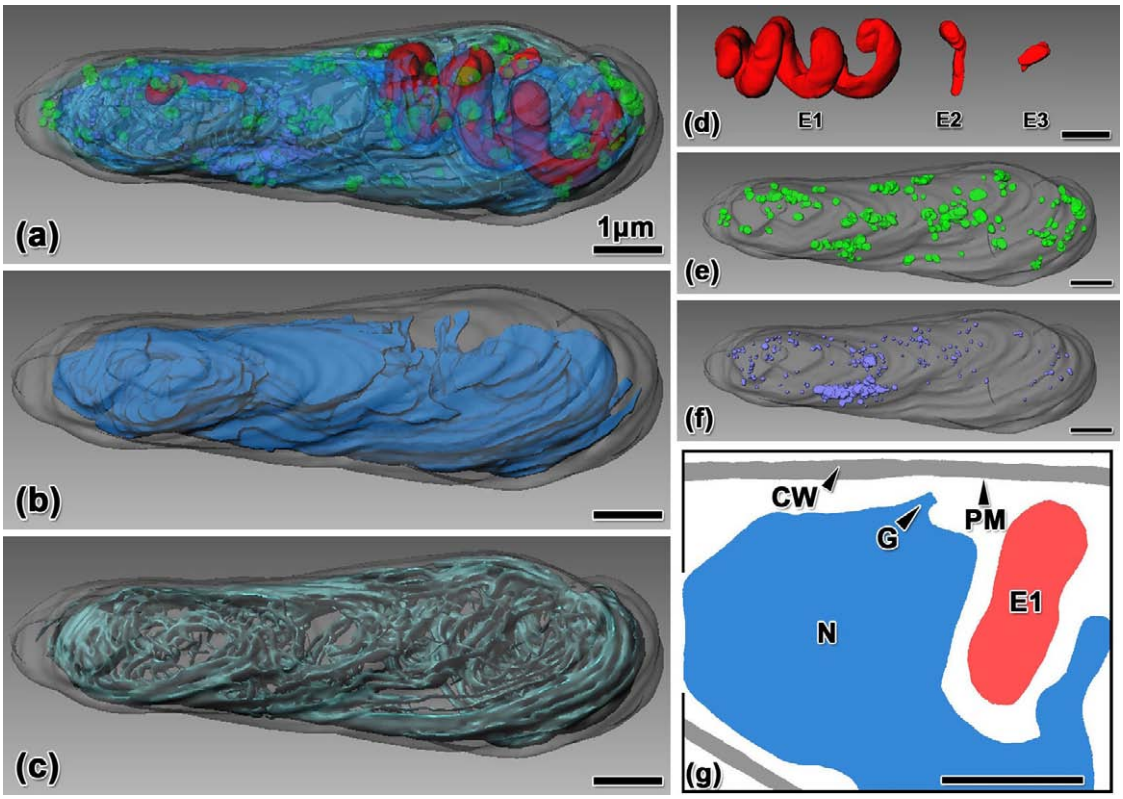

Fig. 14. The three dimensional reconstruction of *P. myojinensis*. (a) The whole cell. (b) The nucleoid. (c) The cytomembrane system of the host cell. (d) The endosymbionts. (e) The distribution of vacuoles in the host cell. (f) The distribution of the small granulated electron-transparent materials in the host cell. (g) Trace image of Fig. 13i showing how the nucleoid region (N) was defined by the inner most cytomembrane (nucleoid membrane) (From Yamaguchi et al. (2012) with permission).

ultrastructure of deep-sea microorganisms at high-resolutions with minimal disturbance of their natural morphologies.

DISCOVERY OF A "MYOJIN PARAKARYOTE"

We prepared 420 specimen blocks by this new CF-FS method, made serial ultrathin sections for all specimens, stained with uranyl acetate and lead citrate, and observed them with an electron microscope. After one year of sectioning and observation, we found a yeast-like microorganism several microns in size with a cell wall (Fig. 11; Yamaguchi et al., 2012).

However, this microorganism was found to lack a nucleus enclosed by a double membrane and mitochondria (Fig. 12). Instead, it had "endosymbionts" with bacteria-like morphology consisting of ribosomes and fibrous nucleoids but no cell wall (Fig. 13a, b). The "nucleoid" of the host cell had a highly irregular shape and occupied most of the host cytoplasm (Figs. 11 and 12). It consisted of fibrous material (DNA) and ribosomes (Fig. 13g). Interestingly, the "nucleoid" was different from both the true nucleoids of prokaryotes and the true nuclei of eukaryotes in that it was enclosed by single-layer membrane, which we refer to as the "nucleoid membrane" (Figs. 11, 13g and 13i). The nucleoid membrane was not a closed membrane system but was interrupted by gaps (Fig. 13i) through which the nucleoid region was connected

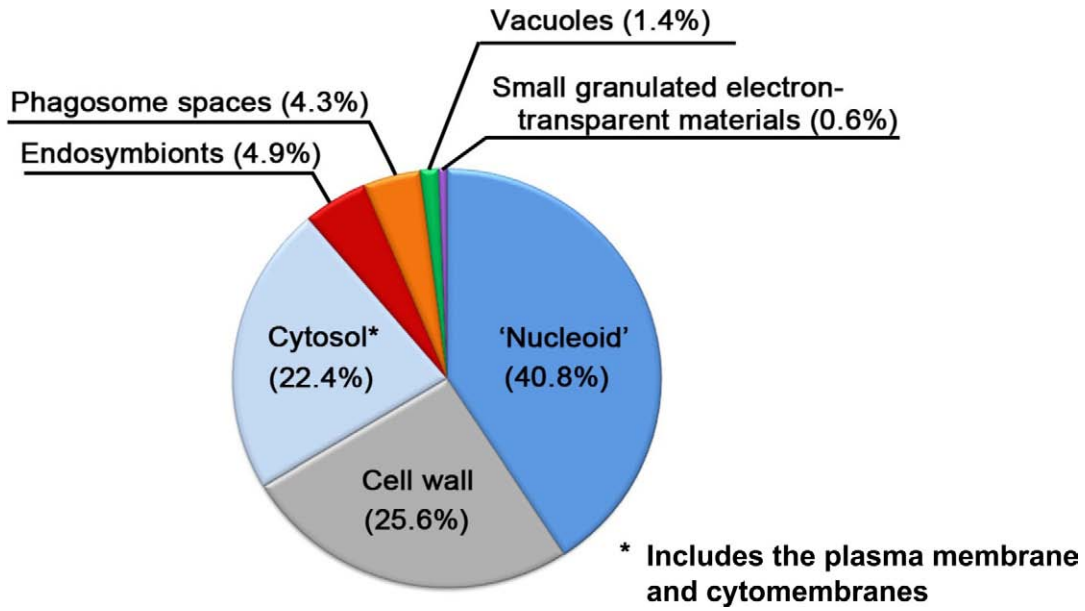

Fig. 15. The volumetric proportions of the cell components in *P. myojinensis* (From Yamaguchi et al. (2012) with permission).

to the cytoplasm, and was notably different from the nuclear envelopes of eukaryotic cells that are made of closed double membranes.

The cell wall consisted of one layer and had a thickness of 80–120 nm (Fig. 13g). The plasma membrane appeared to be a typical three leaflet structure of electron-dense, electron-transparent, and electron-dense material (Fig. 13h) and had a thickness of 19.4 ± 3.9 nm. The cell lacked mitochondria, chloroplasts, a nucleolus, plastids, Golgi apparatuses, peroxisomes, centrioles, spindle pole bodies, and microtubules.

P. myojinensis was 10 μm in length and 3 μm in diameter. By 3D reconstruction from the 67 complete serial sections and structome analysis, we found that the four putative endosymbionts

Table 1. The structomes of *P. myojinensis*, *S. cerevisiae*, *E. dermatitidis*, and *E. coli*

		Parakaryon myojinensis	*Saccharomyces cerevisiae*	*Exophiala dermatitidis*	*Escherichia coli*
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 mito-chondria	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	
Reference		Yamaguchi et al., 2012	Yamaguchi et al., 2011a	Biswas et al., 2003	Pilavtepe-Çelic et al., 2008

This table is modified from Table 2 of Yamaguchi et al. (2012).

apparent in the sectioned image in Fig. 11 (labeled E) were actually different parts of the one large spiral endosymbiont (E1) (Fig. 14a and 14d) and there were a total of three endosymbionts in the cell. The other two endosymbionts (E2 and E3; Fig. 14d) were both rod-shaped and small, together being only around one tenth the volume of the large endosymbiont.

The "nucleoid" of the host occupied 41% of the cell volume (Fig. 15) and was surrounded by a complicated cytomembrane system (Fig. 14c), which occupied 1.7 times the area of the plasma membrane. There were about 100 small vacuoles in the cell (Fig. 14e), which occupied 1.4% of the cell volume (Fig. 15). The cell also contained small granulated electron-transparent materials that occupied 0.6% of the cell volume (Fig. 15) (Fig. 14f), and are typically considered to be storage materials (Biswas et al., 2003). The cytosol, including the plasma membrane, cytomembranes, and ribosomes, occupied 22% of the cell volume (Fig. 15).

MORPHOLOGY OF *PARAKARYON MYOJINENSIS*

It is interesting to compare structomes for *P. myojinensis*, a pair of eukaryotic yeasts (*Saccharomyces cerevisiae* and *Exophiala dermatitidis* (black yeast)), and a prokaryote (*Escherichia coli*) (Table 1). *P. myojinensis* is more than 100 times larger than *E. coli*, three times larger than *S. cerevisiae*, and 1.5 times larger than *E. dermatitidis* (Table 1). The size of prokaryotes is typically confined to a few micrometers because their metabolism is dependent on the diffusion of molecules. Exceptions to this general rule are allowed by special conditions and adaptations such as extraordinarily large resource containing vacuoles, which form the bulk of the cellular volume in *Thiomargarita namibiensis*, or a nutrient-rich environment, highly-folded cell membrane (providing increased surface area for diffusion), and polyploidy (multiple copies of a gene enable the production of its product in different areas of the cell) of *Epulopiscium fishelsoni* (Schulz and Jørgensen, 2001). Because *P. myojinensis* exceeds the normal size for prokaryotes but lacks large vacuoles, a rich environment, or a highly-folded cell membrane, the organism likely has some kind of transport system within the cell; for example, cytoskeleton molecules like actin. We do not know what kind of intracellular transport system this organism has and further study is necessary, however the size and complexity of the "nucleoid" and the cytomembrane system are suggestive of their potential involvement.

This "nucleoid" of *P. myojinensis* occupies more than 40% of the cell volume, whereas the

Table 2. Features of *Parakaryon myojinensis*

1	Cell size	Much larger than ordinary prokaryotes; more than 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 more than 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

This table is modified from Table 3 of Yamaguchi et al. (2012).

nuclei of the yeasts occupy only 7–11% of the cell volumes (Table 1). The endosymbionts in *P. myojinensis* occupy about 5% of the cell volume, a percentage which is in between the mitochondrial volumes in *S. cerevisiae* (2%) and *E. dermatitidis* (10%) (Table 1). The presence of a giant endosymbiont is reminiscent of the presence of a giant mitochondrion in *S. cerevisiae* (Yamaguchi et al., 2011a). The endosymbionts themselves of *P. myojinensis* might fuse or divide during the cell cycle as do the mitochondria of yeast cells. Further study is needed to clarify the nature of the symbiosis between the host and the endosymbionts. Table 2 summarizes the features of *P. myojinensis*.

POSSIBLE ORIGINS OF THE *P. MYOJINENSIS* SPECIMEN

One possibility for the presence of putative "endosymbionts" in a larger cell is predatory or parasitic bacteria living within a prokaryotic host (Guerrero et al., 1986; Larkin et al., 1990). These parasitic bacteria present as intact within the host cells: they show dense cytoplasms, maintain their original rod shapes, and have cell walls. However, in these cases, the cytoplasm of the host cell becomes less dense and shows irregular morphology and thus appears to be degraded by the internal bacteria.

If *P. myojinensis* is a similar case of a host with parasites/predators, the specimen is a snapshot of a short-term predatory interaction. This scenario is unlikely to be the case because of the following three reasons.

First, unlike the reported bacterial parasite-host interactions mentioned above, *P. myojinensis* contained multiple endosymbionts of varying morphology (Fig. 14d). It is unlikely that multiple bacteria of different species attacked a host at the same time. Also, the endosymbionts in *P. myojinensis* lack a cell wall and it is unlikely that a very small bacterium without a cell wall (E3 in Fig. 14d) would be able to successfully attack a prokaryote that had a cell wall. Therefore these endosymbionts cannot have recently entered the host cell as independently living bacteria.

Second, the cytoplasms of both the endosymbionts and the host show intact cellular structures, so no digestion in either host or endosymbionts seems to have occurred. Thus it appears that both the host and the endosymbionts are in good condition and the symbiosis is a long-term interaction.

Third, if *P. myojinensis* is a snapshot of a predator-prey interaction, there must be dense populations of hosts because bacterial predators cannot survive for long between hosts. However, after 14 years, and more than 12,000 micrographs of microorganisms from the deep-sea off the coast of Japan, only one microorganism like *P. myojinensis* was found. This suggests that *P. myojinensis* lives at extremely low densities and is therefore unlikely to be an interaction between predators/parasites and a host.

Instead, *P. myojinensis* seems to be a stable species that originated through an endosymbiotic event in the past involving a larger prokaryote and smaller bacteria as discussed by Margulis (1970). Potentially, *P. myojinensis* could even be a conservative descendent of the transitional lineage between prokaryotes and eukaryotes, however the likelihood of that is low and depends a great deal on how readily bacterial endosymbionts are incorporated into larger cells. At the time of the endosymbiotic event the ancestor of *P. myojinensis* probably had no cell wall, otherwise it would have been unlikely to be able to engulf the bacteria in the first place. *P. myojinensis* currently has a complicated cytomembrane system and a phagosome-like organelle that contains endosymbionts thus it seems likely that its ancestor had the ability to engulf free-living bacteria. The cell wall of *P. myojinensis* most likely formed at some point in time after the endosymbiotic process was complete. Also, the lack of cell walls in the endosymbionts of *P. myojinensis* is likely a derived

characteristic, as they probably had cell walls like other prokaryotes at the beginning of the endosymbiotic event. The cell walls of the endosymbionts must have been lost during a long-term symbiosis as they were no longer needed for protection and would have likely interfered with the benefits of symbiosis.

It is fervently hoped that genetic and biochemical work will be possible in the future when another specimen of *P. myojinensis* is found to enable estimates of its phylogenetic position and the timing of its endosymbiotic event.

IMPLICATIONS FOR THEORIES OF MITOCHONDRIAL AND NUCLEAR DEVELOPMENT

According to the endosymbiosis theory, the ancestor of mitochondria is believed to be an α-proteobacterium. There are several lines of evidence for this theory. 1) Mitochondria have their own DNA (Nass, 1969). 2) They have a double membrane structure (Nass, 1969). 3) They have 70S ribosomes that are similar to bacterial ribosomes and different from eukaryotic ribosomes (Nass, 1969). 4) They multiply within the cell by division (Kuroiwa et al., 1977). 5) The genome of *Rickttsia prowazekii* (an α-proteobacterium) was found to be similar to the mitochondrial genome (Anderson et al., 1998).

The relationship between *P. myojinensis* and its endosymbionts 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 an example that shows another endosymbiotic event that lends support to the hypothesis that the highly-derived mitochondria in eukaryotes could indeed have evolved from bacteria.

The origin of the eukaryote nucleus is another mystery to which *P. myojinensis* may provide clues. There are currently several hypotheses vying for dominance (Lake and Rivera, 1994; Martin, 1999, 2005; Pennisi, 2004). The eukaryote nucleus could have evolved gradually by the development and elaboration of an inner cytomembrane system (Cavalier-Smith, 1988; Nakamura and Hase, 1990), however it could also have been engendered by fusion or symbiosis between multiple prokaryotes (Hartman, 1984; Moreira and López-García, 1998; Horiike et al., 2001).

The "nucleoid" of *P. myojinensis* is not a true nucleus because it does not contain chromatin (DNA associated with histone proteins), which is a hallmark of eukaryotic nuclear organization. Instead the "nucleoid" contains naked DNA fibers, much like the prokaryotic nucleoid. However, true prokaryotic nucleoids are not surrounded by membranes and that of *P. myojinensis* is surrounded by a single layered membrane with scattered gaps. Furthermore, this membrane differs from the eukaryotic nuclear membrane, which is double-layered and complete. This nucleoid membrane could be a form of primitive nuclear membrane. If this is the case, it follows that nuclear membranes could have evolved from the cytomembranes of prokaryotes that had developed inner membrane systems.

There is still a question about whether a nucleus was formed when before mitochondrial ancestors started the process of endosymbiosis (Roger, 1999; Gray et al., 1999; Poole and Penny, 2006; Martijin and Ettema, 2013). 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 a fully formed and differentiated nucleus was not necessary before eubacteria could start to be integrated within the prokaryote host cell. Thus, the formation of the eukaryotic nucleus might not have been linked in any way to the transformation of bacteria into mitochondria.

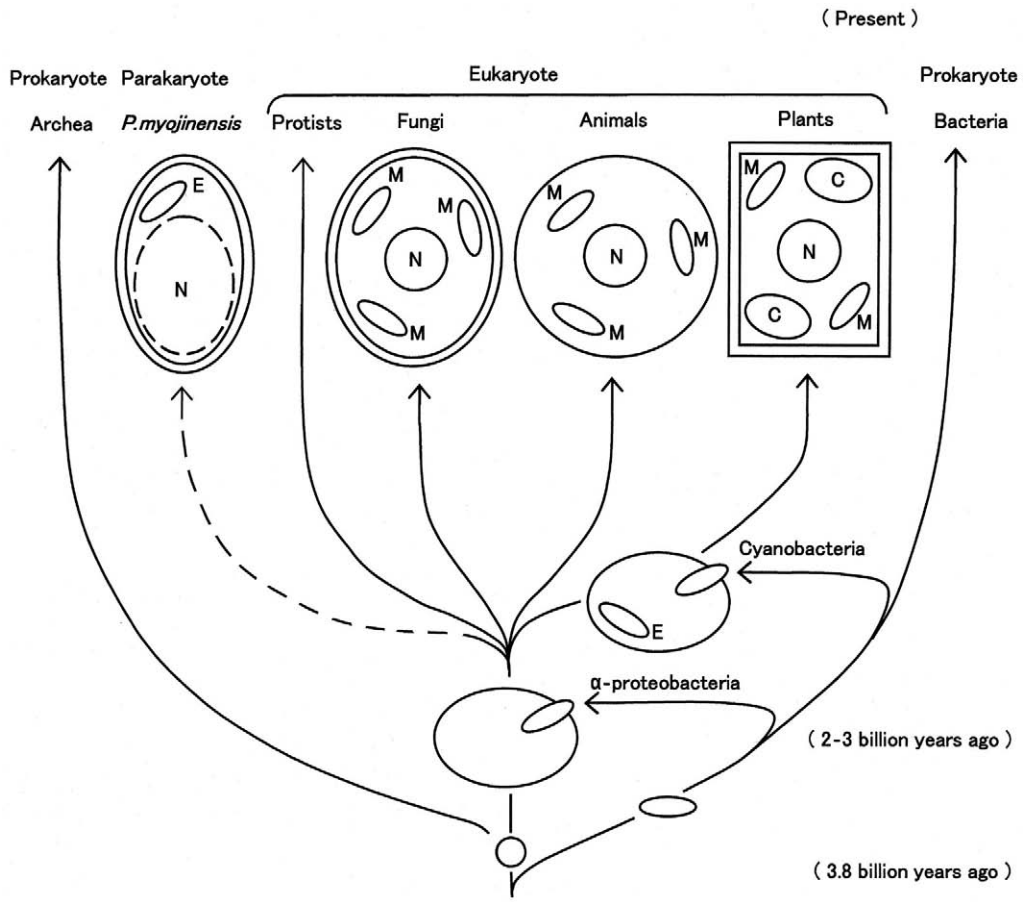

Fig. 16. A model of eukaryotic origins and a possible position of *P. myojinensis*. C, chloroplast; E, endosymbiont; M, mitochondrion; N, nucleus or nucleoid.

A MODEL OF EUKARYOTE ORIGINS

Fig. 16 shows a model of eukaryote origins and a plausible evolutionary position of *P. myojinensis* partly adapted from Whittaker (1969) and Woese *et al.* (1990). Primitive Archea with no cell wall may have engulfed α-proteobacteria by phagocytosis and become the ancestor of protists, fungi, animals, plants, and the parakaryote. The ancestor of plants may have engulfed cyanobacteria at a later time to become modern plants. Cell walls likely arose independently in the plant, fungi, and parakaryote lineages after the endosymbiosis process was complete.

At this point, the phylogenic position of the parakaryote is a matter of pure conjecture. Further genetic studies and biochemical analysis are necessary to elucidate the nature and phylogenetic position of the parakaryote as these are impossible to perform on specimens prepared for electron microscopy. Whatever the true position of the parakaryote in the tree of life, the existence of an apparently transitional life form between prokaryote and eukaryote provides a useful model of what the ancestor to all eukaryotic life could have been like and thus how the transition from prokaryote to eukaryote could have proceeded. It is likely that the cellular physiology and functioning of the

parakaryote are as unique as is its morphology and by providing a third perspective could potentially give a much deeper understanding of cellular mechanisms in general.

THE PROBLEMS AND POTENTIAL OF DEEP-SEA MICROORGANISM STUDIES

There are a few studies of newly discovered deep-sea microorganisms that were characterized morphologically, genetically and/or biochemically after being cultured in the lab (e.g. Takishita et al., 2007; Wu et al., 2014). However, culturing practices are always biased towards certain types of microorganisms with particular tolerances. Considering the fact that standard methods fail to successfully culture most microbes (Pace, 1997), most organisms are overlooked by these methods. Our strategy of direct observation of individual microorganisms 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.

Standard methods of identifying microorganisms through culture and genetic and biochemical characterization are some of the greatest successes of modern biology. However, the natural bias inherent to cell culture techniques severely limits the exploration of unique microorganisms from extreme environments or with unusual requirements. Rather than ignoring these microorganisms and their potentially paradigm-altering structures and adaptations, we believe that careful preparation and morphological characterization can be a useful and important first step in discovering and describing new and unique microorganisms. To do so effectively, we developed the CF-FS method to observe the natural morphology of microorganisms at high resolution by using freeze-substitution electron microscopy (Yamaguchi et al., 2011b). To identify different microorganisms morphologically, we used a serial ultrathin sectioning technique to conduct structome analysis (Yamaguchi et al., 2009, 2011a). These methods enabled us to observe and record a variety of microorganisms from the deep sea, many of which exhibit unusual morphologies. These unusual morphologies are likely mirrored by unusual biochemistry and evolutionary histories and may hold clues to important questions in evolutionary biology, such as the transition from prokaryote to eukaryote, the origins of mitochondria and nuclei, and the origins of centrioles, spindle pole bodies, flagella, and other organelles. The deep sea is an extremely stable environment in which there might be very little selective pressure for change and low levels of competition, leading to still surviving "living fossils" that may retain features long absent from more typical lineages in more "normal" environments (Yamaguchi et al., 2012; Yamaguchi and Worman, 2012; Yamaguchi, 2013).

PERSPECTIVES ON FUTURE RESEARCH

As discussed above, it is difficult to culture deep-sea microorganisms under laboratory conditions. Yet, it is important to obtain genetic data for each microorganism observed with an electron microscope to clarify its phylogenetic position. This presents a tremendous problem because, generally, electron microscopy specimens are fixed with chemicals, embedded in a resin, thinly sliced, stained with heavy metals, and radiated by an electron beam for observation. These harsh treatments must be avoided if the DNA is to remain intact for analysis.

Matching up morphology with phylogenetics would be possible if the same individual cell were able to be used for both types of analysis. This could be achieved by using serial sections of the target microorganism. That is, the specimen is freeze-substituted with ethanol only, embedded, thinly sliced, photographed by electron microscope, and the target is mapped. The next section is cut thick for light microscopy, the target micro-

organism is isolated by laser microdissection, and the DNA/RNA is sequenced. In this technique, since the DNA/RNA of the target microorganism is never treated with chemicals like osmium tetroxide and never irradiated by electron beams, it could be used for sequencing. We are now starting experiments, using the yeast *Saccharomyces* as a model, to develop techniques that make it possible to sequence ribosomal RNA of a microorganism that is also observed under an electron microscope. If this technique is perfected, research on deep-sea microorganisms would advance significantly. This technique would be also useful for other research where the microorganisms cannot be cultured. As it stands now, the partnership between morphology and genetics of microbes lags far behind that of multicellular organisms, but hopefully, this will rapidly change in the near future.

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