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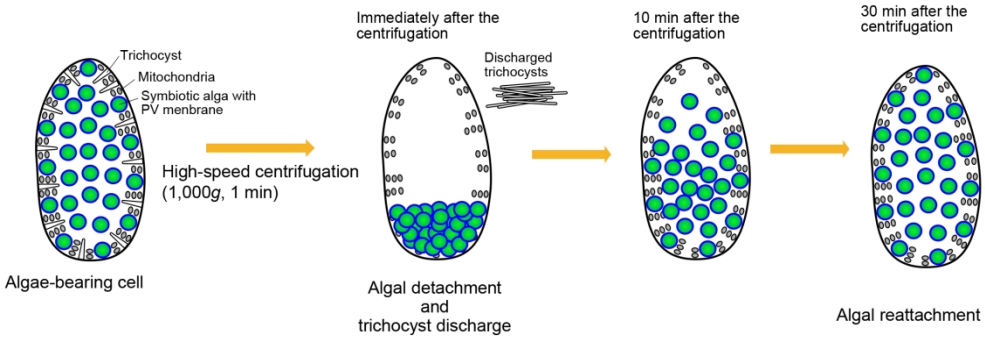

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Role of host ciliate *Paramecium bursaria* mitochondria and trichocysts for symbiotic *Chlorella variabilis* attachment beneath the host cell cortex

Mitochondria and trichocysts of the ciliate *Paramecium bursaria*

One sentence summary: Although the host mitochondria of ciliate *Paramecium bursaria* are near symbiotic algae during reattachment, this does not mean that the algae can exist if mitochondria are present, indicating some localization bias within the host cell.

Abstract

Symbiotic *Chlorella variabilis* is encased in the perialgal vacuole (PV) membrane of ciliate *Paramecium bursaria*. The PV membrane is stably anchored below the host cell cortex by adhesion to host mitochondria. Host trichocysts, which are defensive organelles against predators, are present in the mitochondria and PV membrane vicinity. The mechanism by which PV attaches beneath the host cell cortex remains unknown. When *P. bursaria* is centrifuged at high speed, the symbiotic algae are displaced from the host cell cortex and concentrate at the posterior end. When centrifugation is stopped, the dislocated algae reattach beneath the host cell cortex with fast cytoplasmic streaming. The densities of mitochondria and trichocysts before and after centrifugation were compared using indirect immunofluorescence microscopy with monoclonal antibodies. Almost all trichocysts were shed by high-speed centrifugation, but

dislocated algae could reattach even in the absence of trichocysts. In contrast, host mitochondria were unaffected in localization and number, and the dislocated algae also reattached. These findings suggest trichocysts are unnecessary for algal re-localization and that mitochondria are colocalized with the algae. However, many mitochondria were also present in the cell's anterior region without symbiotic algae. Therefore, not all areas with mitochondria contained algae, but there was a localization bias within the host cell.

Keywords: Algal reattachment, *Chlorella variabilis*, Endosymbiosis, Mitochondria, *Paramecium bursaria*, Trichocyst

Introduction

Paramecium bursaria, a freshwater ciliate, is a symbiotic organism that establishes endosymbiotic relationships with *Chlorella* spp. Each symbiotic alga is enclosed in a perialgal vacuole (PV) derived from the host digestive vacuole (DV), which protects the alga from lysosomal fusion (Gu *et al.* 2002; Karakashian and Rudzinska 1981). This relationship between ciliates and algae is mutualistic; the host cell supplies algae with nitrogen and oxygen (Albers *et al*. 1982, 1985; Reisser 1976, 1980), whereas the algae provide the host with photosynthetic products (Brown *et al*. 1974; Reisser 1986) and CO_2 (Reisser 1980). However, both *P. bursaria* and symbiotic algae can live without their partners. The re-establishment of endosymbiosis between algae-free (removed) *P. bursaria* cells and symbiotic *Chlorella* cells isolated from algae-bearing hosts can be

induced (Kodama and Fujishima 2010). In addition to their original symbiotic algae, algae-free *P. bursaria* cells can be reinfected with bacteria and yeast that are retained in the cytoplasm (Görtz 1982; Watanabe *et al*. 2022). The nuclear genomes of the symbiotic *Chlorella variabilis* (Blanc *et al*. 2010) and the host *P. bursaria* have been explored (Cheng *et al*. 2020); these organisms are now considered models for endosymbiosis research.

Using pulse-labeling and chasing of algae-free paramecia for 1.5 min with symbiotic algae that were isolated from algae-bearing *P. bursaria*, four important cytological events have been identified. These events are necessary for the establishment of endosymbiosis and the timing of each mechanism during the infection process (Kodama and Fujishima 2010). 1) Within 3 min of algal mixing, part of the algae has resistance to the host's lysosomal digestive enzymes in the DVs. 2) Within 30 min of mixing, algae in the DV begin budding from the DV membrane into the cytoplasm. 3) Within 15 min of budding from the DV, the DV membrane enclosing a single green *Chlorella* differentiates into a PV membrane. 4) The PV membrane translocates beneath the host cell cortex.

The PV appears to localize near the host mitochondria and trichocysts. In previous studies, monoclonal antibodies against *P. bursaria* trichocysts and mitochondria were obtained; both mitochondrial and trichocyst densities of algae-bearing *P. bursaria* were significantly lower than those of algae-free cells (Kodama and Fujishima 2011, 2022). These results indicate that symbiotic algae compete for their attachment sites with preexisting trichocysts and mitochondria, and algae have the ability to ensure algal

attachment sites beneath the host cell cortex (Kodama and Fujishima 2011). Furthermore, high-speed centrifugation can induce rapid algal detachment from the host cell cortex and concentrates the algae in the posterior end of the host cell (Kodama and Fujishima 2013). Within 10 min of centrifugation, the detached algae recover their original positions by host rapid cytoplasmic streaming. This algal reattachment was inhibited when host cytoplasmic streaming was arrested by nocodazole. In nocodazole-treated cells, approximately 5 h was required for complete algal recovery, and the host cytoplasmic streaming had been resumed at that time. These results demonstrated that adhesion of the PV beneath the host cell cortex can be repeatedly induced and that host cytoplasmic streaming facilitates the recovery of algal attachment. However, the mechanism by which the PV attaches beneath the host cell cortex remains unknown. In this study, to investigate the mechanisms of symbiotic algal adhesion, the distribution of mitochondria and trichocysts during reattachment was examined by immunofluorescence microscopy using monoclonal antibodies against the mitochondria and trichocysts. Furthermore, mitochondria of the anterior part of the host without symbiotic algae were also observed.

Materials and methods

Organism cultivation

Paramecium bursaria strain Yad1g1N cells (syngen B1 or R3, mating type I) harboring the symbiotic *Chlorella variabilis* strain 1N were used (Kodama and Fujishima 2009, 2011). *Paramecium* cells were cultivated in red pea (*Pisum sativum*) extract culture

medium (Tsukii *et al.* 1995) with a modified Dryl's solution (Dryl 1959; KH_2PO_4 was used instead of $NaH_2PO_4 \cdot 2H_2O$) and inoculated with *Klebsiella aerogenes* (ATCC 35028) one day before use. The cultures were in the early stationary phase of growth one day after the final feeding. All cells used in this study were in this phase. Cultivation was performed in test tubes (18 mm × 180 mm) at 25 ± 1°C under fluorescent lighting at 20–30 μmol photons $m^{-2}s^{-1}$ using an incandescent lamp.

Transmission electron microscopy (TEM)

Algae-bearing *P. bursaria* were pre-fixed with 2% glutaraldehyde and prepared for TEM, as described previously (Kodama *et al.* 2011). The paramecia embedded in Spurr's resin (1969) were sectioned (70 nm thickness) using an ultramicrotome (Reichert Ultracut S; Leica Microsystems, Vienna, Austria) with a diamond knife, mounted on nickel mesh grids, and stained with lead citrate (Reynolds 1963). The sections were observed using TEM (CM120; Philips) at 80 kV.

High-speed centrifugation of *P. bursaria* cells

Algae-bearing *P. bursaria* cells were centrifuged at high speed (Kodama and Fujishima 2011). *P. bursaria* cells at a density of 5×10^3 cells/mL were placed in a 1.5 mL microcentrifuge tube. The tube was then centrifuged using a fixed-angle rotor at 1000 × *g* for 1 min at 25 ± 1° C (Model 3740; Kubota Corporation, Tokyo, Japan). Before, immediately after (approx. 3 min), 10 min, and 30 min after the centrifugation, aliquots of *Paramecium* cells were air-dried on cover glasses (4.5 mm × 24 mm) and fixed with

4% (w/v) paraformaldehyde dissolved in phosphate-buffered saline (PBS) (137 mM NaCl, 2.68 mM KCl, 8.1 mM $NaHPO_4 \cdot 12H_2O$, 1.47 mM KH_2PO_4, pH 7.2) for 10 min at 4°C.

Indirect immunofluorescence microscopy

The cover glasses with the fixed *P. bursaria* cells were washed with PBS containing 0.05% (v/v) Tween 20 (PBST) for 10 min at 4°C. The cells on the cover glasses were treated with a culture supernatant of hybridoma cells containing either monoclonal antibodies mAb5A11E2 against trichocysts of *P. bursaria* (Kodama and Fujishima 2011) or mAb2B8A8H1 against mitochondria of *P. bursaria* (Kodama and Fujishima 2022) overnight at 4°C, washed with PBS, and treated with Alexa Fluor 488 conjugated goat anti-mouse IgG (Molecular Probes Inc., Eugene, OR, USA) diluted 1000-fold with PBS for 2 h at 25°C. The cover glasses were then washed with PBS and observed under a differential interference contrast microscope and fluorescence microscope (BX53; Olympus Corp., Tokyo, Japan). Images were acquired using an Olympus DP74 system and analyzed using Olympus cellSens Dimension software.

Statistical analysis

Data were analyzed using the Mann-Whitney U-test in R. Reproducibility of the data was confirmed by three independent experiments.

Results

Transmission electron microscopy (TEM) of algae-bearing *P. bursaria*

Algae-bearing paramecia were observed using TEM (Fig. 1). As shown by the arrowhead in Fig. 1, we observed mitochondria that attach to the PVs, as shown in previous studies (Fujishima and Kodama 2012; Song *et al.* 2017). In contrast, the trichosyst membrane did not attach to the PV membrane (black arrow in Fig. 1).

Effects of high-speed centrifugation on distribution of trichocysts in algae-bearing *P. bursaria* cells

Figure 2A shows the results obtained by indirect immunofluorescence microscopy after high-speed centrifugation of algae-bearing *P. bursaria* cells using monoclonal antibodies against trichocysts. Before centrifugation, symbiotic algae were shown distributed throughout the host cell by differential interference contrast microscopy, and immunofluorescence showed that the trichocysts of the algae-bearing cells were localized as a ring surrounding the algae, as shown in a previous study (Kodama and Fujishima 2011). Immediately after centrifugation, the symbiotic algae dislocated and accumulated in the posterior region of *Paramecium*. As for trichocysts, immunofluorescence was hardly observed in the centrifuged cells. When cells are stimulated, trichocysts discharge their contents in milliseconds (Adoutte 1988). Therefore, most trichocysts are discharged via high-speed centrifugation. In fact, we observed many discharged trichocysts around the *Parameciucm* cells (Fig. 2B). The monoclonal antibodies also distinguished after discharge, coinciding with findings in a

previous study (Kodama and Fujishima 2011) (Fig. 2B, IM). Ten minutes after centrifugation, the algae completely localized beneath the host cortex, and few trichocysts were observed by immunofluorescence microscopy. Thirty minutes after centrifugation, trichocysts were barely observed. In fact, it was previously reported that when the trichocysts are removed by treatment with lysozyme, regeneration of the mature trichocysts begins at 3 h, even in the presence of lysozyme (Kodama and Fujishima 2009). Therefore, it seems that 30 min are not sufficient for the recovery of trichocysts. These results clearly support previous studies (Kodama and Fujishima 2009) that the symbiotic algae do not need trichocysts to attach beneath the host cell cortex.

Figure 2C shows the immunofluorescence intensity of *P. bursaria* cells before (x-bar, T-B), immediately after (x-bar, T-Ia), 10 min (x-bar, T-10 min), and 30 min (x-bar, T-30 min) after centrifugation. The immunofluorescence intensity of the centrifuged cells decreased drastically and did not increase until 30 min after centrifugation. These quantitative data correspond well with the images shown in Fig. 2A.

Effects of high-speed centrifugation on distribution of mitochondria in algae-bearing *P. bursaria* cells

We examined the effect of high-speed centrifugation on host mitochondria by immunofluorescence microscopy using the same method described above (Fig. 3). Before centrifugation, immunofluorescence of mitochondria was observed around the symbiotic algae. Immediately after centrifugation (approximately 3 min), the symbiotic

algae were dislocated, as shown above. Immunofluorescence was observed throughout the cytoplasm. Note that the cortical mitochondrial immunofluorescence became strong after centrifugation. This phenomenon may be due to better accessibility for visualization of the labeling after detachment of the PV from the cortex. Ten and 30 min after centrifugation, symbiotic algae were distributed throughout the cell because of algal relocation. Immunofluorescence of mitochondria was also observed around the symbiotic algae. These observations suggest that symbiotic algae do not need trichocysts for their attachment but are colocalized with mitochondria, as shown by TEM observations (Fig. 1).

Figure 3B shows the immunofluorescence intensity of algae-free *P. bursaria* cells before (x-bar, M-B), immediately after (x-bar, M-Ia), 10 min (x-bar, M-10 min), and 30 min (x-bar, M-30 min) after centrifugation. There was almost no change in immunofluorescence intensity, and no significant difference was observed between the data for each time point. These quantitative data correspond well with the images shown in Fig. 3A.

Mitochondrial distribution of host anterior cortex

Kodama (2013) investigated the symbiotic algal distribution of 14 strains of *P. bursaria*. As a result, all strains had symbiotic algae at the ventral, dorsal, and posterior cortex, and some cells did not have symbiotic algae at the anterior cortex. This phenomenon is not strain-, syngen-, or mating-type specific. Only 35% of the strain Yad1g1N cells, also used in this experiment, had symbiotic algae at their anterior

cortex. This paper also reported that the artificial trichocyst-discharge experiments by the treatment of lysozyme clarified that trichocysts of the anterior cortex are difficult to remove. Although the high-speed centrifugation experiment clearly showed that trichocysts are unnecessary for algal reattachment, trichocyst-discharge difficulty of the anterior cortex may be related to the restrict enrichment of the large PVs.

On the other hand, host mitochondria remained unchanged in localization by high-speed centrifugation. This indicated that the presence of mitochondria may be related for algal adhesion. To determine whether the reason for the algal difficulty in adhering to the host anterior cortex is due to the low mitochondrial density in this area, we examined whether there were differences in the number and distribution of mitochondria between cells with and without symbiotic algae in their anterior part using indirect immunofluorescence microscopy with the monoclonal antibodies against host mitochondria.

Figure 4 shows differential interference contrast microscopy images of *P. bursaria* cells with (Algae+) and without (Algae-) symbiotic algae in the anterior end and immunofluorescence images of those cells using mitochondrial monoclonal antibodies. Note that mitochondria are present in the anterior end without symbiotic algae as well as in the anterior end with symbiotic algae. This observation shows that not all areas with mitochondria had algae, but there was a localization bias within the host cell.

Discussion

Localization of endosymbionts near the host cell cortex is a universal phenomenon; the same phenomenon has been observed in other ciliate -algae or -cyanobacteria endosymbionts, such as *Mayorella viridis*, *Coleps hirtus*, *Coleps spetai*, *Frontonia leucas*, *Malacophrys sphagni*, *Ophrydium versatile*, *Vorticella* sp., *Climacostomum virens*, *Euplotes daidaleos*, *Halteria bifurcata*, *Stentor polymorphus*, and *Stentor niger* (Reisser 1986). Furthermore, the ability to adjust the intracellular symbiont position is likely an important means of optimizing carbon production (Petrou *et al*. 2017).

A previous study showed that the infectivity of *Chlorella* species in *P. bursaria* is based on their ability to localize beneath the host cell membrane after escaping from the host digestive vacuole during the early infection process (Kodama and Fujishima 2007). Furthermore, this algal attachment may be related to the avoidance of host lysosomal digestion or fusion because algal digestion when the symbiotic algae are attached beneath the host cell cortex has not been observed (Kodama and Fujishima unpubl. data).

Acidosomes are organelles responsible for the acidification of DVs before lysosomal fusion and are distributed throughout the cell (Allen 1993; Kodama 2013). High-speed centrifugation can also induce the rapid accumulation of host acidosomes and lysosomes at the anterior end of *Paramecium*. Within 10 min of centrifugation, the accumulated vesicles recover their original positions by host rapid cytoplasmic streaming (Kodama 2013). This observation showed that although acidosomes and lysosomes were dislocated by centrifugation, the mitochondria remained unchanged.

This is probably because acidosomes and lysosomes undergo cytoplasmic streaming, but mitochondria do not.

Figure 5 shows a schematic representation of the algal attachment mechanism beneath the host cell cortex after dislocation via high-speed centrifugation. After the high-speed centrifugation, almost all trichocysts are released out of the cell. The discharged trichocysts were regenerate from the endoplasmic reticulum, pass through the Golgi apparatus, and undergo a maturation process (pretrichocysts) before mature trichocysts are delivered to the cell membrane (Plattner 2017). Pretrichocysts contain a growing mass of electron dense secretory material. The pretrichocysts then elongate while their luminal space is progressively filled with crystallizing secretory materials (Garreau De Loubresse 1993). Because several hours are required for regeneration after trichocyst discharge (Harumoto 2002), no trichocysts are observed in the cells after algal reattachment. This representation can be defined by obtaining monoclonal antibodies against both mitochondria and trichocysts. To the best of our knowledge, this is the first report showing the distribution of host mitochondria and trichocysts during algal relocalization beneath the host cell cortex.

Fast cytoplasmic streaming after high-speed centrifugation of *P. bursaria* is the driving force for algal relocalization. The characteristic feature of rotational cytoplasmic streaming in the genus *Paramecium* (*P. aurelia*, *P. caudatum*, *P. bursaria*, *P. multimicronucleatum,* and *P. calkinsi*) is that the pattern of its route and direction remain constant (Sikora 1981). Various factors, such as cell division, conjugation, temperature, and osmotic pressure, are known to affect cytoplasmic streaming in

Paramecium (Sikora 1981), but the detachment of the symbiotic algae by high-speed centrifugation is also a factor. In *P. bursaria–Chlorella* endosymbiosis, their cell cycle pace is guaranteed to be synchronous (Takahashi 2016). Takahashi *et al*. (2007) found that *P. bursaria* controls the proliferation of endosymbiotic algae through host-cell-cycle-dependent cytoplasmic streaming. Thus, host cytoplasmic streaming plays a major role in maintaining the number and cellular localization of symbiotic algae in *P. bursaria* cells.

The mitochondria of the host *P. bursaria* are close to the PV membrane (Fig. 1), and their number and function are altered by the presence or absence of symbiotic algae (Kodama and Fujishima 2022). Therefore, host mitochondria and symbiotic algae are expected to be strongly associated with each other. We assumed that the presence of mitochondria was essential for the existence of symbiotic algae; however, the results in Fig. 4 contradict this expectation. Mitochondria and endoplasmic reticulum (ER) are contact at ER-Mitochondria Contact Sites (EMCS)s. Lipids and Ca^{2+} synthesized in the ER are transported to mitochondria through the EMCSs (Kornmann *et al*. 2009; Rizzuto *et al*. 1998). Furthermore, EMCs is also important in reactive oxygen species (ROS) production (Booth *et al*. 2016) and autophagosome formation (Hamasaki *et al*. 2013). Song *et al.* (2017) reported that the host ER is also involved in organizing intracellular algal symbiosis in the cytoplasm. The relationship between host mitochondria and symbiotic algae and the relationship between other cellular organelles, including the host endoplasmic reticulum, should be examined in detail in future studies.

The exposure of *P. bursaria* cells to ultraviolet and photosynthetically active radiation is known to induce symbiotic algal dislocation, moving to the posterior cell region (Summerer *et al.* 2009). The displaced algae relocate to a "normal" distribution when host cells are transferred to a medium without ultraviolet radiation conditions. The mechanisms of algal displacement and relocation remain unclear (Summerer *et al.* 2009). A dislocation of *Chlorella* symbionts has also been described in the ciliate *Pelagodileptus trachelioides* (Butkay 2004). Although this phenomenon appears to be a stress reaction followed by host cytolysis, the details remain unknown. The method of artificial algal detachment and subsequent rapid reattachment by high-speed centrifugation of algae-bearing *P. bursaria* may contribute to understanding the mechanism of symbiotic algal localization in a variety of host species.

Although the mechanism of PV localization beneath the host cell cortex is still unknown, the docking or distribution mechanism of mitochondria and uninserted trichocysts in other ciliate species, such as *Paramecium tetraurelia,* have been reported. Aufderheide (1977) reported that, in the subcortical regions of *P. tetraurelia*, mitochondria and uninserted (undocked) trichocysts display saltatory motility with individual characteristics, making them distinguishable from each other and from cellular cyclosis. The saltatory motion of trichocysts is implicated as a means of transporting new trichocysts from the cytoplasm to their ultimate location in the cellular cortex. In addition, the saltatory motion may be a factor in the intracellular distribution of the mitochondria. The mechanism of trichocyst docking at the *Paramecium* cell membrane has been reported in detail (Plattner 2017; Plattner *et al.* 1982). Before

trichocyst docking, mature trichocysts move through the subcortical region by cyclosis (Sikora 1981). Then, they approach, in a saltatory manner, tip first, a docking site at the cell membrane (Aufderheide 1978); Thereby, trichocysts are guided by the same "hand." This entity is a microtubule emanating from a nearby ciliary basal body (Plattner *et al.* 1982), which always is situated ~ 0.5 to 1 μm from a docking site proper. Petrou *et al.* (2017) found that the intracellular algal symbionts within the large photosymbiotic foraminifera *Marginopora vertebralis* exhibit phototactic behavior and the phototactic movement of the symbionts is accomplished by the host through rapid actin-mediated relocation of the symbionts deeper into the cavities within the calcite skeletons. Thus, the host cytoskeleton plays a major role in the migration of host cytosolic organelles and symbiotic algae. In order to study the involvement of the cytoskeleton in the process of PV localization, the cell cortex of algae-bearing *P. bursaria* cells was observed in detail by TEM. However, no cytoskeletal structure that seems to be related to PV localization has been found until now (Kodama unpub. Data).

Adhesion just below the host cell surface is a universal phenomenon found not only in cell organelles and symbiotic algae but also in pathogenic bacteria. Protists are potential natural hosts of various bacterial species, including pathogens. *Paramecium caudatum* could be a natural reservoir of *Legionella pneumophila* (Watanabe *et al.* 2016), and *P. bursaria* could be a potential host of *Francisella novicida* (Watanabe *et al.* 2022). *Francisella novicida* is a facultative intracellular pathogen and a causative agent of tularemia (Ellis *et al.* 2002). Recently, Watanabe *et al.* (2022) found that *F. novicida* (strain U112) cells, which were wrapped with the host DV membrane, localized beneath the host cell cortex. This indicates that both symbiotic algae and

bacteria can attach beneath the host cell cortex. Therefore, elucidation of the mechanism of localization beneath the host cell cortex using *P. bursaria* is expected to contribute to the development of not only symbiotic biology but also infectious disease research.

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Figure Legends

Figure 1. (A) Transmission electron microscopy of the symbiotic algae and the surrounding trichocyst and mitochondria. The magnified image of the mitochondria is shown in **(B)**. Note that mitochondrion is attached to a PV membrane (PVm, arrowhead). tc, trichocyst; tcm, trichocyst membrane; mt, mitochondrion; bb, basal body; PVm, PV membrane; a, symbiotic algae.

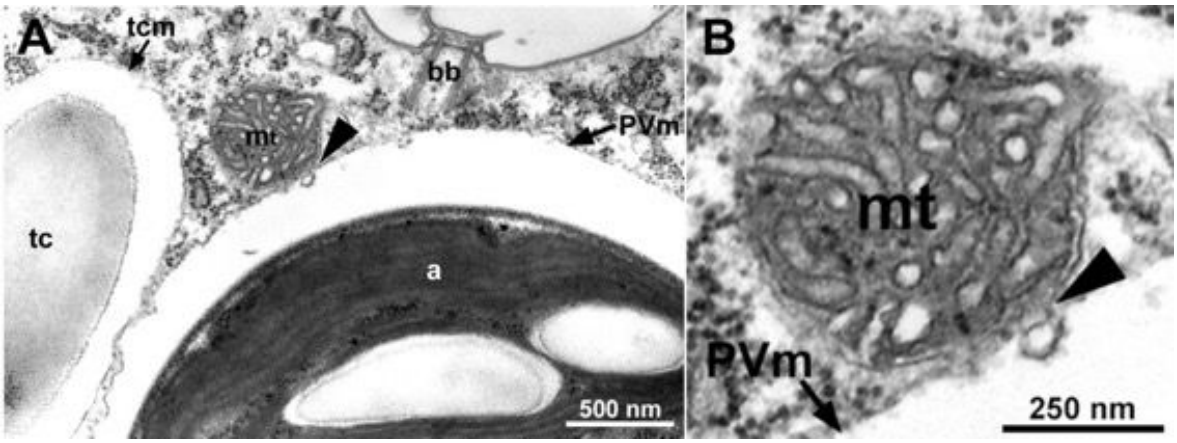
Figure 2. (A) Indirect immunofluorescence micrographs of the high-speed centrifuged algae-bearing *Paramecia* with the monoclonal antibody against trichocysts. Before centrifugation, the symbiotic algae localized throughout the host cell were observed by differential interference contrast (DIC) microscopy. Immunofluorescence (IM) is observed in the whole cell. Immediately after the centrifugation, the symbiotic algae were dislocated and concentrated at the posterior~~anterior~~ side~~side~~. Ten min after the centrifugation, the dislocated algae distributed throughout the cell by host cytoplasmic streaming. Thirty min after the centrifugation, algal re-localization was completed. IM is hardly observed in the centrifuged cell because of discharge of trichocysts induced by the high-speed centrifugation as shown in **(B)**. Immunofluorescence is still not observed even though the algal re-localized. Ma, macronucleus. **(B)** DIC and IM micrographs of discharged trichocysts from a certain *P. bursaria* cell. The trichocyst is completely free from the *P. bursaria*. Trichocysts are colorless and transparent, making them difficult to observe by the DIC microscopy, but easy to observe by the IM. **(C)** Immunofluorescence intensity of the centrifuged cells decreased drastically, and did not increase. ~~Twelve to 20 *Paramecium* cells were observed.~~ Error bars show standard deviation (SD). Asterisks indicate significant differences (Mann-Whitney U test, ***P < 0.001, Twelve to 20 *Paramecium* cells were observed).

Figure 3. (A) Indirect immunofluorescence micrographs of the high-speed centrifuged algae-bearing *Paramecia* with the monoclonal antibody against mitochondria. Photographs were taken by focusing on mitochondria showing strong immunofluorescence during each observation period. Before the centrifugation, the symbiotic algae were observed throughout the host cell were observed by differential interference contrast (DIC) microscopy. Immunofluorescence (IM) is also observed in the whole cell. Immediately after the centrifugation, the symbiotic algae were dislocated and concentrated at the posterior side. IM is still observed in the whole cell. Algal

reattachment was complete within 30 minutes and no change in mitochondrial fluorescence was observed. Immediately after the high-speed centrifugation, the symbiotic algae localized to the posterior side. Thus, only the posterior side of the *Paramecia* becomes thicker and stiffer due to the presence of many algae, making it really difficult to focus on the posterior side. Therefore, the focus of the immediate after is different from that of the other three (Before, 10 min, and 30 min) periods. Ma, macronucleus. **(B)** Immunofluorescence intensity of the centrifuged cells did not change before and after the centrifugation, and there was no significant differences (Mann-Whitney U test, Ten to 12 *Paramecium* cells were observed) ~~were observed~~. ~~Ten to 12 *Paramecium* cells were observed.~~ Error bars show standard deviation (SD).

Figure 4. Indirect immunofluorescence micrographs with the monoclonal antibody against mitochondria. *Paramecium bursaria* cells with (Algae+) and without (Algae-) symbiotic algae on their anterior were observed by differential interference contrast (DIC) microscopy. Immunofluorescence (IM) indicates that host mitochondria is present in areas with and without symbiotic algae.

Figure 5. A schematic diagram of changes in localization of symbiotic algae with PV membrane, host mitochondria, and trichocysts before and after high-speed centrifugation. Before the centrifugation, numerous host mitochondria and trichocysts are attached beneath the host cell cortex, with symbiotic algae settled between them. After the centrifugation, the symbiotic algae detach from the host cell cortex, immediately accumulating at the posterior end of the host cell. At the same time, almost all trichocysts are released out of the cell, but mitochondria remain anchored. The symbiotic algae gradually reattach by host cytoplasmic streaming, which is complete within 30 min. At that time, the algae attach between mitochondria, but trichocysts are not required for the algal attachment.



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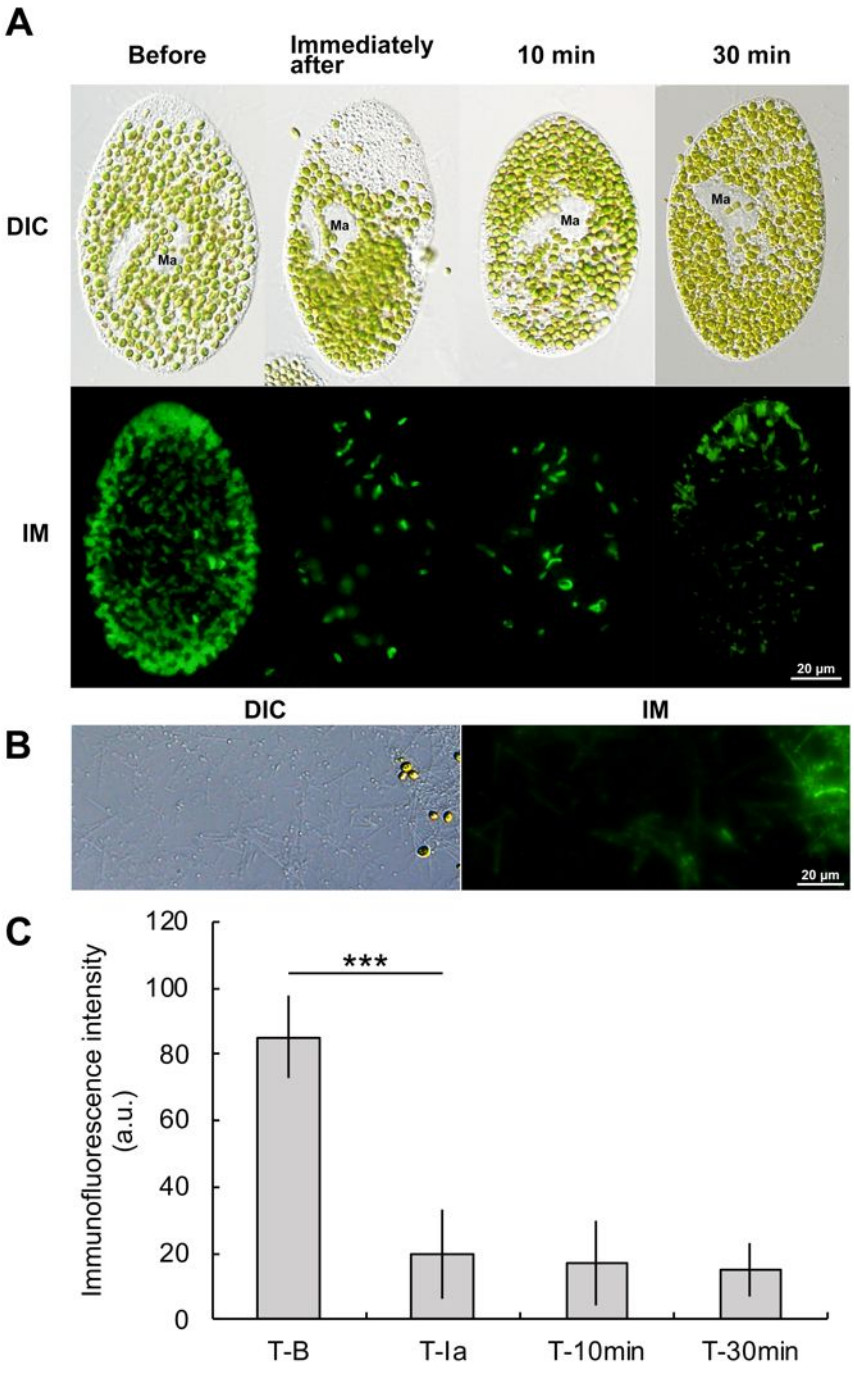

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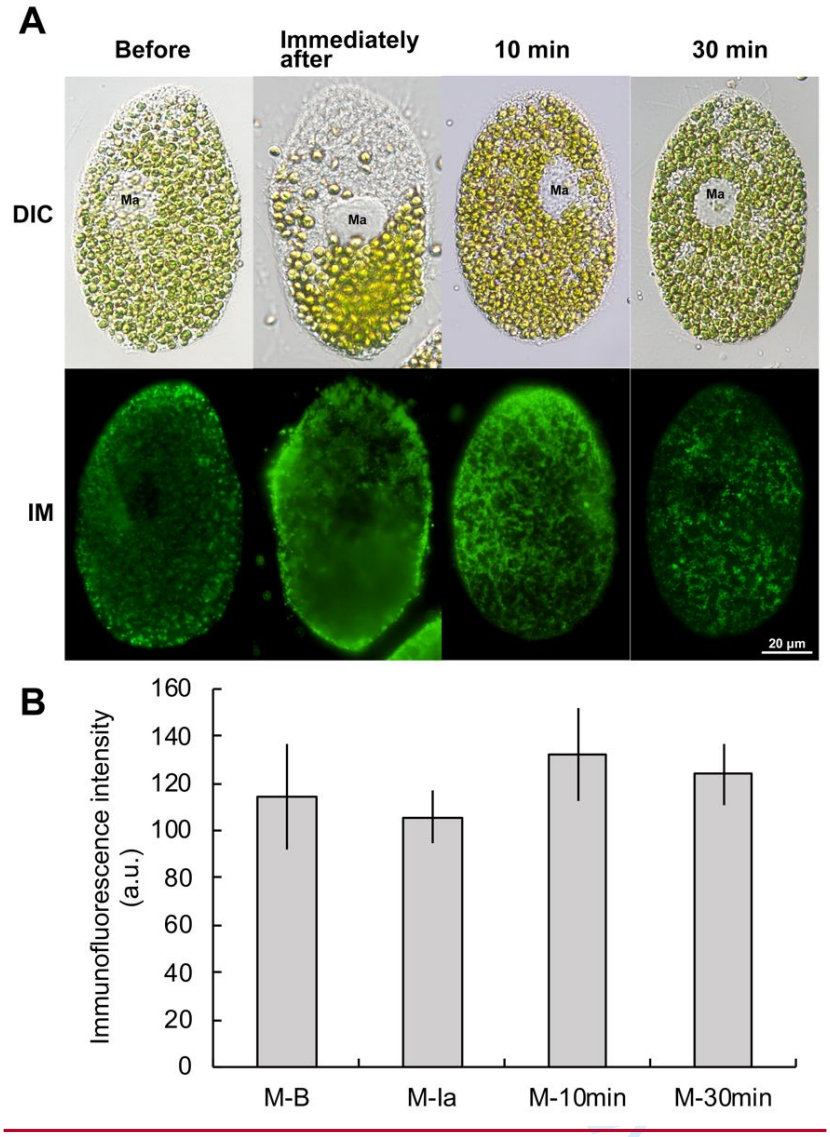

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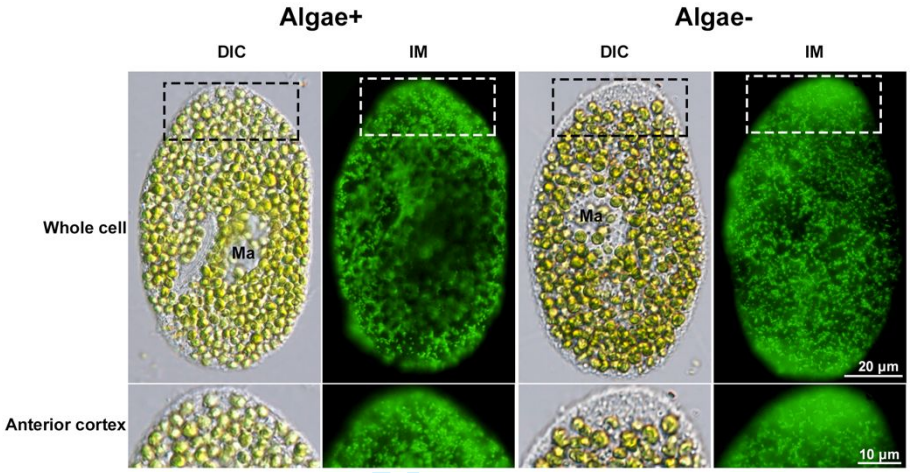

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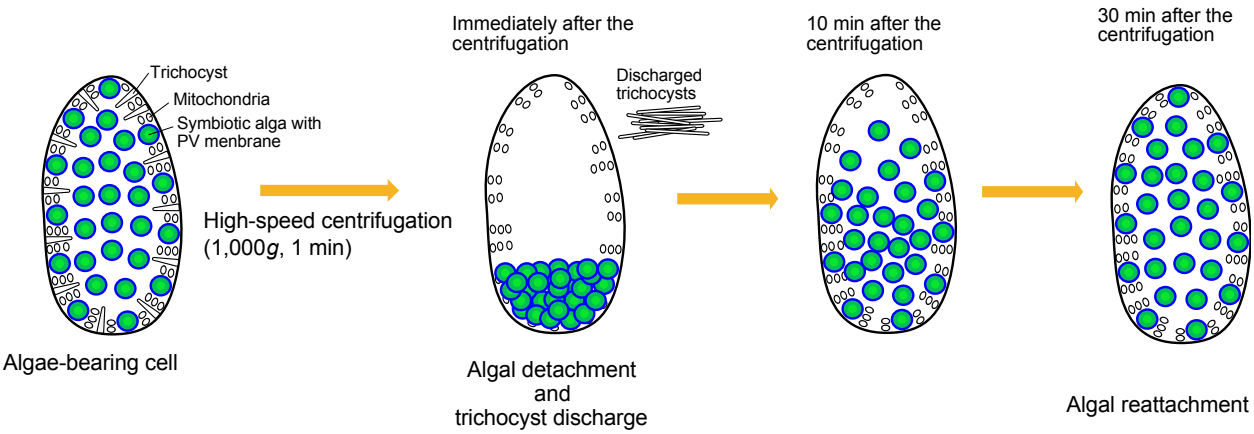

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Role of host ciliate *Paramecium bursaria* mitochondria and trichocysts for symbiotic *Chlorella variabilis* attachment beneath the host cell cortex

Mitochondria and trichocysts of the ciliate *Paramecium bursaria*

One sentence summary: Although the host mitochondria of ciliate *Paramecium bursaria* are near symbiotic algae during reattachment, this does not mean that the algae can exist if mitochondria are present, indicating some localization bias within the host cell.

Abstract

Symbiotic *Chlorella variabilis* is encased in the perialgal vacuole (PV) membrane of ciliate *Paramecium bursaria*. The PV membrane is stably anchored below the host cell cortex by adhesion to host mitochondria. Host trichocysts, which are defensive organelles against predators, are present in the mitochondria and PV membrane vicinity. The mechanism by which PV attaches beneath the host cell cortex remains unknown. When *P. bursaria* is centrifuged at high speed, the symbiotic algae are displaced from the host cell cortex and concentrate at the posterior end. When centrifugation is stopped, the dislocated algae reattach beneath the host cell cortex with fast cytoplasmic streaming. The densities of mitochondria and trichocysts before and after centrifugation were compared using indirect immunofluorescence microscopy with monoclonal antibodies. Almost all trichocysts were shed by high-speed centrifugation, but

dislocated algae could reattach even in the absence of trichocysts. In contrast, host mitochondria were unaffected in localization and number, and the dislocated algae also reattached. These findings suggest trichocysts are unnecessary for algal re-localization and that mitochondria are colocalized with the algae~~, unlike mitochondria~~. However, many mitochondria were also present in the cell's anterior region without symbiotic algae. Therefore, not all areas with mitochondria contained algae, but there was a localization bias within the host cell.

Keywords: Algal reattachment, *Chlorella variabilis*, Endosymbiosis, Mitochondria, *Paramecium bursaria*, Trichocyst

Introduction

Paramecium bursaria, a freshwater ciliate, is a symbiotic organism that establishes endosymbiotic relationships with *Chlorella* spp. Each symbiotic alga is enclosed in a perialgal vacuole (PV) derived from the host digestive vacuole (DV), which protects the alga from lysosomal fusion (Gu *et al.* 2002; Karakashian and Rudzinska 1981). This relationship between ciliates and algae is mutualistic; the host cell supplies algae with nitrogen and oxygen (Albers *et al*. 1982, 1985; Reisser 1976, 1980), whereas the algae provide the host with photosynthetic products (Brown *et al*. 1974; Reisser 1986) and CO_2 (Reisser 1980). However, both *P. bursaria* and symbiotic algae can live without their partners. The re-establishment of endosymbiosis between algae-free (removed) *P. bursaria* cells and symbiotic *Chlorella* cells isolated from algae-bearing hosts can be

induced (Kodama and Fujishima 2010). In addition to their original symbiotic algae, algae-free *P. bursaria* cells can be reinfected with bacteria and yeast that are retained in the cytoplasm (Görtz 1982; Watanabe *et al*. 2022). The nuclear genomes of the symbiotic *Chlorella variabilis* (Blanc *et al*. 2010) and the host *P. bursaria* have been explored (Cheng *et al*. 2020); these organisms are now considered models for endosymbiosis research.

Using pulse-labeling and chasing of algae-free paramecia for 1.5 min with symbiotic algae that were isolated from algae-bearing *P. bursaria*, four important cytological events have been identified. These events are necessary for the establishment of endosymbiosis and the timing of each mechanism during the infection process (Kodama and Fujishima 2010). 1) Within 3 min of algal mixing, part of the algae has resistance to the host's lysosomal digestive enzymes in the DVs. 2) Within 30 min of mixing, algae in the DV begin budding from the DV membrane into the cytoplasm. 3) Within 15 min of budding from the DV, the DV membrane enclosing a single green *Chlorella* differentiates into a PV membrane. 4) The PV membrane translocates beneath the host cell cortex.

The PV appears to localize near the host mitochondria and trichocysts. In previous studies, monoclonal antibodies against *P. bursaria* trichocysts and mitochondria were obtained; both mitochondrial and trichocyst densities of algae-bearing *P. bursaria* were significantly lower than those of algae-free cells (Kodama and Fujishima 2011, 2022). These results indicate that symbiotic algae compete for their attachment sites with preexisting trichocysts and mitochondria, and algae have the ability to ensure algal

attachment sites beneath the host cell cortex (Kodama and Fujishima 2011). Furthermore, high-speed centrifugation can induce rapid algal detachment from the host cell cortex and concentrates the algae in the posterior end of the host cell (Kodama and Fujishima 2013). Within 10 min of centrifugation, the detached algae recover their original positions by host rapid cytoplasmic streaming. This algal reattachment was inhibited when host cytoplasmic streaming was arrested by nocodazole. In nocodazole-treated cells, approximately 5 h was required for complete algal recovery, and the host cytoplasmic streaming had been resumed at that time. These results demonstrated that adhesion of the PV beneath the host cell cortex can be repeatedly induced and that host cytoplasmic streaming facilitates the recovery of algal attachment. However, the mechanism by which the PV attaches beneath the host cell cortex remains unknown. In this study, to investigate the mechanisms of symbiotic algal adhesion, the distribution of mitochondria and trichocysts during reattachment was examined by immunofluorescence microscopy using monoclonal antibodies against the mitochondria and trichocysts. Furthermore, mitochondria of the anterior part of the host without symbiotic algae were also observed.

Materials and methods

Organism cultivation

Paramecium bursaria strain Yad1g1N cells (syngen B1 or R3, mating type I) harboring the symbiotic *Chlorella variabilis* strain 1N were used (Kodama and Fujishima 2009, 2011). *Paramecium* cells were cultivated in red pea (*Pisum sativum*) extract culture

medium (Tsukii *et al.* 1995) with a modified Dryl's solution (Dryl 1959;) (KH_2PO_4 was used instead of $NaH_2PO_4 \cdot 2H_2O$) and inoculated with *Klebsiella aerogenes* (ATCC 35028) one day before use. The cultures were in the early stationary phase of growth one day after the final feeding. All cells used in this study were in this phase. Cultivation was performed in test tubes (18 mm × 180 mm) at 25 ± 1°C under fluorescent lighting at 20–30 µmol photons $m^{-2}s^{-1}$ using an incandescent lamp.

Transmission electron microscopy (TEM)

Algae-bearing *P. bursaria* were pre-fixed with 2% glutaraldehyde and prepared for TEM, as described previously (Kodama *et al.* 2011). The paramecia embedded in Spurr's resin (1969) were sectioned (70 nm thickness) using an ultramicrotome (Reichert Ultracut S; Leica Microsystems, Vienna, Austria) with a diamond knife, mounted on nickel mesh grids, and stained with lead citrate (Reynolds 1963). The sections were observed using TEM (CM120; Philips) at 80 kV.

High-speed centrifugation of *P. bursaria* cells

Algae-bearing *P. bursaria* cells were centrifuged at high speed (Kodama and Fujishima 2011). *P. bursaria* cells at a density of 5 ×10^3 cells/mL were placed in a 1.5 mL microcentrifuge tube. The tube was then centrifuged using a fixed-angle rotor at 1000 × *g* for 1 min at 25 ± 1° C (Model 3740; Kubota Corporation, Tokyo, Japan). Before, immediately after (approx. 3 min), 10 min, and 30 min after the centrifugation, aliquots of *Paramecium* cells were air-dried on cover glasses (4.5 mm × 24 mm) and fixed with

4% (w/v) paraformaldehyde dissolved in phosphate-buffered saline (PBS) (137 mM NaCl, 2.68 mM KCl, 8.1 mM $NaHPO_4 \cdot 12H_2O$, 1.47 mM KH_2PO_4, pH 7.2) for 10 min at 4°C.

Indirect immunofluorescence microscopy

The cover glasses with the fixed *P. bursaria* cells were washed with PBS containing 0.05% (v/v) Tween 20 (PBST) for 10 min at 4°C. The cells on the cover glasses were treated with a culture supernatant of hybridoma cells containing either monoclonal antibodies mAb5A11E2 against trichocysts of *P. bursaria* (Kodama and Fujishima 2011) or mAb2B8A8H1 against mitochondria of *P. bursaria* (Kodama and Fujishima 2022) overnight at 4°C, washed with PBS, and treated with Alexa Fluor 488 conjugated goat anti-mouse IgG (Molecular Probes Inc., Eugene, OR, USA) diluted 1000-fold with PBS for 2 h at 25°C. The cover glasses were then washed with PBS and observed under a differential interference contrast microscope and fluorescence microscope (BX53; Olympus Corp., Tokyo, Japan). Images were acquired using an Olympus DP74 system and analyzed using Olympus cellSens Dimension software.

Statistical analysis

Data were analyzed using the Mann-Whitney U-test in R. Reproducibility of the data was confirmed by three independent experiments.

Results

Transmission electron microscopy (TEM) of algae-bearing *P. bursaria*

Algae-bearing paramecia were observed using TEM (Fig. 1). As shown by the arrowhead in Fig. 1, we observed mitochondria that attach to the PVs, as shown in previous studies (Fujishima and Kodama 2012; Song *et al.* 2017). In contrast, the trichosyst membrane did not attach to the PV membrane (black arrow in Fig. 1).

Effects of high-speed centrifugation on distribution of trichocysts in algae-bearing *P. bursaria* cells

Figure 2A shows the results obtained by indirect immunofluorescence microscopy after high-speed centrifugation of algae-bearing *P. bursaria* cells using monoclonal antibodies against trichocysts. Before centrifugation, symbiotic algae were shown distributed throughout the host cell by differential interference contrast microscopy, and immunofluorescence showed that the trichocysts of the algae-bearing cells were localized as a ring surrounding the algae, as shown in a previous study (Kodama and Fujishima 2011). Immediately after centrifugation, the symbiotic algae dislocated and accumulated in the posterior region of *Paramecium*. As for trichocysts, immunofluorescence was hardly observed in the centrifuged cells. When cells are stimulated, trichocysts discharge their contents in milliseconds (Adoutte 1988). Therefore, most trichocysts are discharged via high-speed centrifugation. In fact, we observed many discharged trichocysts around the *Parameciucm* cells (Fig. 2B). The monoclonal antibodies also distinguished after discharge, coinciding with findings in a

previous study (Kodama and Fujishima 2011) (Fig. 2B, IM). Ten minutes after centrifugation, the algae completely localized beneath the host cortex, and few trichocysts were observed by immunofluorescence microscopy. Thirty minutes after centrifugation, trichocysts were barely observed. In fact, it was previously reported that when the trichocysts are removed by treatment with lysozyme, regeneration of the mature trichocysts begins at 3 h, even in the presence of lysozyme (Kodama and Fujishima 2009). Therefore, it seems that 30 min are~~is~~ not sufficient for the recovery of trichocysts. These results clearly support previous studies (Kodama and Fujishima 2009) that the symbiotic algae do not need trichocysts to attach beneath the host cell cortex.

Figure 2C shows the immunofluorescence intensity of *P. bursaria* cells before (x-bar, T-B), immediately after (x-bar, T-Ia), 10 min (x-bar, T-10 min), and 30 min (x-bar, T-30 min) after centrifugation. The immunofluorescence intensity of the centrifuged cells decreased drastically and did not increase until 30 min after centrifugation. These quantitative data correspond well with the images shown in Fig. 2A.~~These quantitative data correspond well with the results of indirect immunofluorescence microscopy, as shown in Fig. 2A.~~

Effects of high-speed centrifugation on distribution of mitochondria in algae-bearing *P. bursaria* cells

We examined the effect of high-speed centrifugation on host mitochondria by immunofluorescence microscopy using the same method described above (Fig. 3). Before centrifugation, immunofluorescence of mitochondria was observed around the symbiotic algae. Immediately after centrifugation (approximately 3 min), the symbiotic algae were dislocated, as shown above. Immunofluorescence was observed throughout the cytoplasm. Note that the cortical mitochondrial immunofluorescence became strong after centrifugation. This phenomenon may be due to better accessibility for visualization of the labeling after detachment of the PV from the cortex. Ten and 30 min after centrifugation, symbiotic algae were distributed throughout the cell because of algal relocation. Immunofluorescence of mitochondria was also observed around the symbiotic algae. These observations suggest that symbiotic algae do not need trichocysts for their attachment but are colocalized with mitochondria ~~for their attachment~~, as shown by TEM observations (Fig. 1).

Figure 3B shows the immunofluorescence intensity of algae-free *P. bursaria* cells before (x-bar, M-B), immediately after (x-bar, M-Ia), 10 min (x-bar, M-10 min), and 30 min (x-bar, M-30 min) after centrifugation. There was almost no change in immunofluorescence intensity, and no significant difference was observed between the data for each time point. These quantitative data correspond well with the images shown in Fig. 3A~~This quantitative data corresponds well with the results of indirect immunofluorescence microscopy, as shown in Fig. 3A~~.

Mitochondrial distribution of host anterior cortex

Kodama (2013) investigated the symbiotic algal distribution of 14 strains of *P. bursaria*. As a result, all strains had symbiotic algae at the ventral, dorsal, and posterior cortex~~ices~~, and some cells did not have symbiotic algae at the anterior cortex. This phenomenon is not strain-, syngen-, or mating-type specific. Only 35% of the strain Yad1g1N cells, also used in this experiment, had symbiotic algae at their anterior cortex. This paper also reported that the artificial trichocyst-discharge experiments by the treatment of lysozyme clarified that trichocysts of the anterior cortex are difficult to remove. Although the high-speed centrifugation experiment clearly showed that trichocysts are unnecessary for algal reattachment, trichocyst-discharge difficulty of the anterior cortex may be related to the restrict enrichment of the large PVs.~~It is unclear why algae have difficulty adhering to the anterior cortex of their hosts. The high-speed centrifugation experiment clearly showed that trichocysts are unnecessary for algal reattachment.~~

On the other hand, host mitochondria remained unchanged in localization by high-speed centrifugation. This indicated that the presence of mitochondria ~~might~~ may be ~~essential~~ related for algal adhesion. To determine whether the reason for the algal difficulty in adhering to the host anterior cortex is due to the low mitochondrial density in this area, we examined whether there were differences in the number and distribution of mitochondria between cells with and without symbiotic algae in their anterior part using indirect immunofluorescence microscopy with the monoclonal antibodies against host mitochondria.

Figure 4 shows differential interference contrast microscopy images of *P. bursaria* cells with (Algae+) and without (Algae-) symbiotic algae in the anterior end and immunofluorescence images of those cells using mitochondrial monoclonal antibodies. Note that mitochondria are present in the anterior end without symbiotic algae as well as in the anterior end with symbiotic algae. This observation shows that not all areas with mitochondria had algae, but there was a localization bias within the host cell.

Discussion

Localization of endosymbionts near the host cell cortex is a universal phenomenon; the same phenomenon has been observed in other ciliate -algae or -cyanobacteria endosymbionts, such as *Mayorella viridis*, *Coleps hirtus*, *Coleps spetai*, *Frontonia leucas*, *Malacophrys sphagni*, *Ophrydium versatile*, *Vorticella* sp., *Climacostomum virens*, *Euplotes daidaleos*, *Halteria bifurcata*, *Stentor polymorphus*, and *Stentor niger* (Reisser 1986). Furthermore, the ability to adjust the intracellular symbiont position is likely an important means of optimizing carbon production (Petrou *et al*. 2017).

A previous study showed that the infectivity of *Chlorella* species in *P. bursaria* is based on their ability to localize beneath the host cell membrane after escaping from the host digestive vacuole during the early infection process (Kodama and Fujishima 2007). Furthermore, this algal attachment may be related to the avoidance of host lysosomal digestion or fusion because algal digestion when the symbiotic algae are attached beneath the host cell cortex has not been observed (Kodama and Fujishima unpubl. data).

Acidosomes are organelles responsible for the acidification of DVs before lysosomal fusion and are distributed throughout the cell (Allen 1993; Kodama 2013). High-speed centrifugation can also induce the rapid accumulation of host acidosomes and lysosomes at the anterior end of *Paramecium*. Within 10 min of centrifugation, the accumulated vesicles recover their original positions by host rapid cytoplasmic streaming (Kodama 2013). This observation showed that although acidosomes and lysosomes were dislocated by centrifugation, the mitochondria remained unchanged. This is probably because acidosomes and lysosomes undergo cytoplasmic streaming, but mitochondria do not.

Figure 5 shows a schematic representation of the algal attachment mechanism beneath the host cell cortex after dislocation via high-speed centrifugation. After the high-speed centrifugation, almost all trichocysts are released out of the cell. The discharged trichocysts were regenerate from the endoplasmic reticulum, pass through the Golgi apparatus, and undergo a maturation process (pretrichocysts) before mature trichocysts are delivered to the cell membrane (Plattner 2017). Pretrichocysts contain a growing mass of electron dense secretory material. The pretrichocysts then elongate while their luminal space is progressively filled with crystallizing secretory materials (Garreau De Loubresse 1993). Because several hours are required for regeneration after trichocyst discharge (Harumoto 2002), no trichocysts are observed in the cells after algal reattachment. This representation can be defined by obtaining monoclonal antibodies against both mitochondria and trichocysts. To the best of our knowledge, this

is the first report showing the distribution of host mitochondria and trichocysts during algal relocalization beneath the host cell cortex.

Fast cytoplasmic streaming after high-speed centrifugation of *P. bursaria* is the driving force for algal relocalization. The characteristic feature of rotational cytoplasmic streaming in the genus *Paramecium* (*P. aurelia*, *P. caudatum*, *P. bursaria*, *P. multimicronucleatum,* and *P. calkinsi*) is that the pattern of its route and direction remain constant (Sikora 1981). Various factors, such as cell division, conjugation, temperature, and osmotic pressure, are known to affect cytoplasmic streaming in *Paramecium* (Sikora 1981), but the detachment of the symbiotic algae by high-speed centrifugation is also a factor. In *P. bursaria–Chlorella* endosymbiosis, their cell cycle pace is guaranteed to be synchronous (Takahashi 2016). Takahashi *et al*. (2007) found that *P. bursaria* controls the proliferation of endosymbiotic algae through host-cell-~~,~~ cycle-dependent cytoplasmic streaming. Thus, host cytoplasmic streaming plays a major role in maintaining the number and cellular localization of symbiotic algae in *P. bursaria* cells.

The mitochondria of the host *P. bursaria* are close to the PV membrane (Fig. 1), and their number and function are altered by the presence or absence of symbiotic algae (Kodama and Fujishima 2022). Therefore, host mitochondria and symbiotic algae are expected to be strongly associated with each other. We assumed that the presence of mitochondria was essential for the existence of symbiotic algae; however, the results in Fig. 4 contradict this expectation. Mitochondria and endoplasmic reticulum (ER) are contact at ER-Mitochondria Contact Sites (EMCS)s. Lipids and Ca^{2+} synthesized in the

ER are transported to mitochondria through the EMCSs (Kornmann *et al*. 2009; Rizzuto *et al*. 1998). Furthermore, EMCs is also important in reactive oxygen species (ROS) production (Booth *et al*. 2016) and autophagosome formation (Hamasaki *et al*. 2013). Song *et al.* (2017) reported that the host ~~endoplasmic reticulum~~ER is also involved in organizing intracellular algal symbiosis in the cytoplasm. The relationship between host mitochondria and symbiotic algae and the relationship between other cellular organelles, including the host endoplasmic reticulum, should be examined in detail in future studies.

The exposure of *P. bursaria* cells to ultraviolet and photosynthetically active radiation is known to induce symbiotic algal dislocation, moving to the posterior cell region (Summerer *et al.* 2009). The displaced algae relocate to a "normal" distribution when host cells are transferred to a medium without ultraviolet radiation conditions. The mechanisms of algal displacement and relocation remain unclear (Summerer *et al.* 2009). A dislocation of *Chlorella* symbionts has also been described in the ciliate *Pelagodileptus trachelioides* (Butkay 2004). Although this phenomenon appears to be a stress reaction followed by host cytolysis, the details remain unknown. The method of artificial algal detachment and subsequent rapid reattachment by high-speed centrifugation of algae-bearing *P. bursaria* may contribute to understanding the mechanism of symbiotic algal localization in a variety of host species.

Although the mechanism of PV localization beneath the host cell cortex is still unknown, the docking or distribution mechanism of mitochondria and uninserted trichocysts in other ciliate species, such as *Paramecium tetraurelia,* have been reported.

Aufderheide (1977) reported that, in the subcortical regions of *P. tetraurelia*, mitochondria and uninserted (undocked) trichocysts display saltatory motility with individual characteristics, making them distinguishable from each other and from cellular cyclosis. The saltatory motion of trichocysts is implicated as a means of transporting new trichocysts from the cytoplasm to their ultimate location in the cellular cortex. In addition, the saltatory motion may be a factor in the intracellular distribution of the mitochondria. The mechanism of trichocyst docking at the *Paramecium* cell membrane has been reported in detail (Plattner 2017; Plattner *et al.* 1982). Before trichocyst docking, mature trichocysts move through the subcortical region by cyclosis (Sikora 1981). Then, they approach, in a saltatory manner, tip first, a docking site at the cell membrane (Aufderheide 1978); Thereby, trichocysts are guided by the same "hand." This entity is a microtubule emanating from a nearby ciliary basal body (Plattner *et al.* 1982), which always is situated ~ 0.5 to 1 μm from a docking site proper. Petrou *et al.* (2017) found that the intracellular algal symbionts within the large photosymbiotic foraminifera *Marginopora vertebralis* exhibit phototactic behavior and the phototactic movement of the symbionts is accomplished by the host through rapid actin-mediated relocation of the symbionts deeper into the cavities within the calcite skeletons. Thus, the host cytoskeleton plays a major role in the migration of host cytosolic organelles and symbiotic algae. In order to study the involvement of the cytoskeleton in the process of PV localization, the cell cortex of algae-bearing *P. bursaria* cells was observed in detail by TEM. However, no cytoskeletal structure that seems to be related to PV localization has been found until now (Kodama unpub. Data).

Adhesion just below the host cell surface is a universal phenomenon found not only in cell organelles and symbiotic algae but also in pathogenic bacteria. Protists are potential natural hosts of various bacterial species, including pathogens. *Paramecium caudatum* could be a natural reservoir of *Legionella pneumophila* (Watanabe *et al*. 2016), and *P. bursaria* could be a potential host of *Francisella novicida* (Watanabe *et al.* 2022). *Francisella novicida* is a facultative intracellular pathogen and a causative agent of tularemia (Ellis *et al.* 2002). Recently, Watanabe *et al.* (2022) found that *F. novicida* (strain U112) cells, which were wrapped with the host DV membrane, localized beneath the host cell cortex. This indicates that both symbiotic algae and bacteria can attach beneath the host cell cortex. Therefore, elucidation of the mechanism of localization beneath the host cell cortex using *P. bursaria* is expected to contribute to the development of not only symbiotic biology but also infectious disease research.

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Role of host ciliate *Paramecium bursaria* mitochondria and trichocysts for symbiotic *Chlorella variabilis* attachment beneath the host cell cortex

Mitochondria and trichocysts of the ciliate *Paramecium bursaria*

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One sentence summary: Although the host mitochondria of ciliate *Paramecium bursaria* are near symbiotic algae during reattachment, this does not mean that the algae can exist if mitochondria are present, indicating some localization bias within the host cell.

Abstract

Symbiotic *Chlorella variabilis* is encased in the perialgal vacuole (PV) membrane of ciliate *Paramecium bursaria*. The PV membrane is stably anchored below the host cell cortex by adhesion to host mitochondria. Host trichocysts, which are defensive organelles against predators, are present in the mitochondria and PV membrane vicinity. The mechanism by which PV attaches beneath the host cell cortex remains unknown. When *P. bursaria* is centrifuged at high speed, the symbiotic algae are displaced from the host cell cortex and concentrate at the posterior end. When centrifugation is stopped, the dislocated algae reattach beneath the host cell cortex with fast cytoplasmic streaming. The densities of mitochondria and trichocysts before and after centrifugation were compared using indirect immunofluorescence microscopy with monoclonal antibodies. Almost all trichocysts were shed by high-speed centrifugation, but dislocated algae could reattach even in the absence of trichocysts. In contrast, host mitochondria were unaffected in localization and number, and the dislocated algae also reattached. These findings suggest trichocysts are unnecessary for algal re-localization and that mitochondria are colocalized with the algae~~, unlike mitochondria~~. However, many mitochondria were also present in the cell's anterior region without symbiotic

algae. Therefore, not all areas with mitochondria contained algae, but there was a localization bias within the host cell.

Keywords: Algal reattachment, *Chlorella variabilis*, Endosymbiosis, Mitochondria, *Paramecium bursaria*, Trichocyst

Introduction

Paramecium bursaria, a freshwater ciliate, is a symbiotic organism that establishes endosymbiotic relationships with *Chlorella* spp. Each symbiotic alga is enclosed in a perialgal vacuole (PV) derived from the host digestive vacuole (DV), which protects the alga from lysosomal fusion (Gu *et al.* 2002; Karakashian and Rudzinska 1981). This relationship between ciliates and algae is mutualistic; the host cell supplies algae with nitrogen and oxygen (Albers *et al*. 1982, 1985; Reisser 1976, 1980), whereas the algae provide the host with photosynthetic products (Brown *et al*. 1974; Reisser 1986) and CO_2 (Reisser 1980). However, both *P. bursaria* and symbiotic algae can live without their partners. The re-establishment of endosymbiosis between algae-free (removed) *P. bursaria* cells and symbiotic *Chlorella* cells isolated from algae-bearing hosts can be induced (Kodama and Fujishima 2010). In addition to their original symbiotic algae, algae-free *P. bursaria* cells can be reinfected with bacteria and yeast that are retained in the cytoplasm (Görtz 1982; Watanabe *et al*. 2022). The nuclear genomes of the symbiotic *Chlorella variabilis* (Blanc *et al*. 2010) and the host *P. bursaria* have been

explored (Cheng *et al*. 2020); these organisms are now considered models for endosymbiosis research.

Using pulse-labeling and chasing of algae-free paramecia for 1.5 min with symbiotic algae that were isolated from algae-bearing *P. bursaria*, four important cytological events have been identified. These events are necessary for the establishment of endosymbiosis and the timing of each mechanism during the infection process (Kodama and Fujishima 2010). 1) Within 3 min of algal mixing, part of the algae has resistance to the host's lysosomal digestive enzymes in the DVs. 2) Within 30 min of mixing, algae in the DV begin budding from the DV membrane into the cytoplasm. 3) Within 15 min of budding from the DV, the DV membrane enclosing a single green *Chlorella* differentiates into a PV membrane. 4) The PV membrane translocates beneath the host cell cortex.

The PV appears to localize near the host mitochondria and trichocysts. In previous studies, monoclonal antibodies against *P. bursaria* trichocysts and mitochondria were obtained; both mitochondrial and trichocyst densities of algae-bearing *P. bursaria* were significantly lower than those of algae-free cells (Kodama and Fujishima 2011, 2022). These results indicate that symbiotic algae compete for their attachment sites with preexisting trichocysts and mitochondria, and algae have the ability to ensure algal attachment sites beneath the host cell cortex (Kodama and Fujishima 2011). Furthermore, high-speed centrifugation can induce rapid algal detachment from the host cell cortex and concentrates the algae in the posterior end of the host cell (Kodama and Fujishima 2013). Within 10 min of centrifugation, the detached algae recover their

original positions by host rapid cytoplasmic streaming. This algal reattachment was inhibited when host cytoplasmic streaming was arrested by nocodazole. In nocodazole-treated cells, approximately 5 h was required for complete algal recovery, and the host cytoplasmic streaming had been resumed at that time. These results demonstrated that adhesion of the PV beneath the host cell cortex can be repeatedly induced and that host cytoplasmic streaming facilitates the recovery of algal attachment. However, the mechanism by which the PV attaches beneath the host cell cortex remains unknown. In this study, to investigate the mechanisms of symbiotic algal adhesion, the distribution of mitochondria and trichocysts during reattachment was examined by immunofluorescence microscopy using monoclonal antibodies against the mitochondria and trichocysts. Furthermore, mitochondria of the anterior part of the host without symbiotic algae were also observed.

Materials and methods

Organism cultivation

Paramecium bursaria strain Yad1g1N cells (syngen B1 or R3, mating type I) harboring the symbiotic *Chlorella variabilis* strain 1N were used (Kodama and Fujishima 2009, 2011). *Paramecium* cells were cultivated in red pea (*Pisum sativum*) extract culture medium (Tsukii *et al.* 1995) with a modified Dryl's solution (Dryl 1959;) (KH_2PO_4 was used instead of $NaH_2PO_4 \cdot 2H_2O$) and inoculated with *Klebsiella aerogenes* (ATCC 35028) one day before use. The cultures were in the early stationary phase of growth one day after the final feeding. All cells used in this study were in this phase.

Cultivation was performed in test tubes (18 mm × 180 mm) at 25 ± 1°C under fluorescent lighting at 20–30 μmol photons $m^{-2}s^{-1}$ using an incandescent lamp.

Transmission electron microscopy (TEM)

Algae-bearing *P. bursaria* were pre-fixed with 2% glutaraldehyde and prepared for TEM, as described previously (Kodama *et al.* 2011). The paramecia embedded in Spurr's resin (1969) were sectioned (70 nm thickness) using an ultramicrotome (Reichert Ultracut S; Leica Microsystems, Vienna, Austria) with a diamond knife, mounted on nickel mesh grids, and stained with lead citrate (Reynolds 1963). The sections were observed using TEM (CM120; Philips) at 80 kV.

High-speed centrifugation of *P. bursaria* cells

Algae-bearing *P. bursaria* cells were centrifuged at high speed (Kodama and Fujishima 2011). *P. bursaria* cells at a density of 5×10^3 cells/mL were placed in a 1.5 mL microcentrifuge tube. The tube was then centrifuged using a fixed-angle rotor at 1000 × *g* for 1 min at 25 ± 1° C (Model 3740; Kubota Corporation, Tokyo, Japan). Before, immediately after (approx. 3 min), 10 min, and 30 min after the centrifugation, aliquots of *Paramecium* cells were air-dried on cover glasses (4.5 mm × 24 mm) and fixed with 4% (w/v) paraformaldehyde dissolved in phosphate-buffered saline (PBS) (137 mM NaCl, 2.68 mM KCl, 8.1 mM $NaHPO_4 \cdot 12H_2O$, 1.47 mM KH_2PO_4, pH 7.2) for 10 min at 4°C.

Indirect immunofluorescence microscopy

The cover glasses with the fixed *P. bursaria* cells were washed with PBS containing 0.05% (v/v) Tween 20 (PBST) for 10 min at 4°C. The cells on the cover glasses were treated with a culture supernatant of hybridoma cells containing either monoclonal antibodies mAb5A11E2 against trichocysts of *P. bursaria* (Kodama and Fujishima 2011) or mAb2B8A8H1 against mitochondria of *P. bursaria* (Kodama and Fujishima 2022) overnight at 4°C, washed with PBS, and treated with Alexa Fluor 488 conjugated goat anti-mouse IgG (Molecular Probes Inc., Eugene, OR, USA) diluted 1000-fold with PBS for 2 h at 25°C. The cover glasses were then washed with PBS and observed under a differential interference contrast microscope and fluorescence microscope (BX53; Olympus Corp., Tokyo, Japan). Images were acquired using an Olympus DP74 system and analyzed using Olympus cellSens Dimension software.

Statistical analysis

Data were analyzed using the Mann-Whitney U-test in R. Reproducibility of the data was confirmed by three independent experiments.

Results

Transmission electron microscopy (TEM) of algae-bearing *P. bursaria*

Algae-bearing paramecia were observed using TEM (Fig. 1). As shown by the arrowhead in Fig. 1, we observed mitochondria that attach to the PVs, as shown in

previous studies (Fujishima and Kodama 2012; Song *et al.* 2017). In contrast, the trichosyst membrane did not attach to the PV membrane (black arrow in Fig. 1).

Effects of high-speed centrifugation on distribution of trichocysts in algae-bearing *P. bursaria* cells

Figure 2A shows the results obtained by indirect immunofluorescence microscopy after high-speed centrifugation of algae-bearing *P. bursaria* cells using monoclonal antibodies against trichocysts. Before centrifugation, symbiotic algae were shown distributed throughout the host cell by differential interference contrast microscopy, and immunofluorescence showed that the trichocysts of the algae-bearing cells were localized as a ring surrounding the algae, as shown in a previous study (Kodama and Fujishima 2011). Immediately after centrifugation, the symbiotic algae dislocated and accumulated in the posterior region of *Paramecium*. As for trichocysts, immunofluorescence was hardly observed in the centrifuged cells. When cells are stimulated, trichocysts discharge their contents in milliseconds (Adoutte 1988). Therefore, most trichocysts are discharged via high-speed centrifugation. In fact, we observed many discharged trichocysts around the *Parameciucm* cells (Fig. 2B). The monoclonal antibodies also distinguished after discharge, coinciding with findings in a previous study (Kodama and Fujishima 2011) (Fig. 2B, IM). Ten minutes after centrifugation, the algae completely localized beneath the host cortex, and few trichocysts were observed by immunofluorescence microscopy. Thirty minutes after

centrifugation, trichocysts were barely observed. In fact, it was previously reported that when the trichocysts are removed by treatment with lysozyme, regeneration of the mature trichocysts begins at 3 h, even in the presence of lysozyme (Kodama and Fujishima 2009). Therefore, it seems that 30 min are~~is~~ not sufficient for the recovery of trichocysts. These results clearly support previous studies (Kodama and Fujishima 2009) that the symbiotic algae do not need trichocysts to attach beneath the host cell cortex.

Figure 2C shows the immunofluorescence intensity of *P. bursaria* cells before (x-bar, T-B), immediately after (x-bar, T-Ia), 10 min (x-bar, T-10 min), and 30 min (x-bar, T-30 min) after centrifugation. The immunofluorescence intensity of the centrifuged cells decreased drastically and did not increase until 30 min after centrifugation. These quantitative data correspond well with the images shown in Fig. 2A ~~These quantitative data correspond well with the results of indirect immunofluorescence microscopy, as shown in Fig. 2A.~~

Effects of high-speed centrifugation on distribution of mitochondria in algae-bearing *P. bursaria* cells

We examined the effect of high-speed centrifugation on host mitochondria by immunofluorescence microscopy using the same method described above (Fig. 3). Before centrifugation, immunofluorescence of mitochondria was observed around the symbiotic algae. Immediately after centrifugation (approximately 3 min), the symbiotic

algae were dislocated, as shown above. Immunofluorescence was observed throughout the cytoplasm. Note that the cortical mitochondrial immunofluorescence became strong after centrifugation. This phenomenon may be due to better accessibility for visualization of the labeling after detachment of the PV from the cortex. Ten and 30 min after centrifugation, symbiotic algae were distributed throughout the cell because of algal relocation. Immunofluorescence of mitochondria was also observed around the symbiotic algae. These observations suggest that symbiotic algae do not need trichocysts for their attachment but are colocalized with mitochondria ~~for their attachment~~, as shown by TEM observations (Fig. 1).

Figure 3B shows the immunofluorescence intensity of algae-free *P. bursaria* cells before (x-bar, M-B), immediately after (x-bar, M-Ia), 10 min (x-bar, M-10 min), and 30 min (x-bar, M-30 min) after centrifugation. There was almost no change in immunofluorescence intensity, and no significant difference was observed between the data for each time point. These quantitative data correspond well with the images shown in Fig. 3A~~This quantitative data corresponds well with the results of indirect immunofluorescence microscopy, as shown in Fig. 3A~~.

Mitochondrial distribution of host anterior cortex

Kodama (2013) investigated the symbiotic algal distribution of 14 strains of *P. bursaria*. As a result, all strains had symbiotic algae at the ventral, dorsal, and posterior cortex~~ices~~, and some cells did not have symbiotic algae at the anterior cortex. This phenomenon is not strain-, syngen-, or mating-type specific. Only 35% of the strain

Yad1g1N cells, also used in this experiment, had symbiotic algae at their anterior cortex. This paper also reported that the artificial trichocyst-discharge experiments by the treatment of lysozyme clarified that trichocysts of the anterior cortex are difficult to remove. Altough the high-speed centrifugation experiment clearly showed that trichocysts are unnecessary for algal reattachment, trichocyst-discharge difficulty of the anterior cortex may be related to the restrict enrichment of the large PVs. ~~It is unclear why algae have difficulty adhering to the anterior cortex of their hosts. The high-speed centrifugation experiment clearly showed that trichocysts are unnecessary for algal reattachment.~~

On the other hand, host mitochondria remained unchanged in localization by high-speed centrifugation. This indicated that the presence of mitochondria ~~might~~ may be ~~essential~~ related for algal adhesion. To determine whether the reason for the algal difficulty in adhering to the host anterior cortex is due to the low mitochondrial density in this area, we examined whether there were differences in the number and distribution of mitochondria between cells with and without symbiotic algae in their anterior part using indirect immunofluorescence microscopy with the monoclonal antibodies against host mitochondria.

Figure 4 shows differential interference contrast microscopy images of *P. bursaria* cells with (Algae+) and without (Algae-) symbiotic algae in the anterior end and immunofluorescence images of those cells using mitochondrial monoclonal antibodies. Note that mitochondria are present in the anterior end without symbiotic algae as well

as in the anterior end with symbiotic algae. This observation shows that not all areas with mitochondria had algae, but there was a localization bias within the host cell.

Discussion

Localization of endosymbionts near the host cell cortex is a universal phenomenon; the same phenomenon has been observed in other ciliate -algae or -cyanobacteria endosymbionts, such as *Mayorella viridis*, *Coleps hirtus*, *Coleps spetai*, *Frontonia leucas*, *Malacophrys sphagni*, *Ophrydium versatile*, *Vorticella* sp., *Climacostomum virens*, *Euplotes daidaleos*, *Halteria bifurcata*, *Stentor polymorphus*, and *Stentor niger* (Reisser 1986). Furthermore, the ability to adjust the intracellular symbiont position is likely an important means of optimizing carbon production (Petrou *et al*. 2017).

A previous study showed that the infectivity of *Chlorella* species in *P. bursaria* is based on their ability to localize beneath the host cell membrane after escaping from the host digestive vacuole during the early infection process (Kodama and Fujishima 2007). Furthermore, this algal attachment may be related to the avoidance of host lysosomal digestion or fusion because algal digestion when the symbiotic algae are attached beneath the host cell cortex has not been observed (Kodama and Fujishima unpubl. data).

Acidosomes are organelles responsible for the acidification of DVs before lysosomal fusion and are distributed throughout the cell (Allen 1993; Kodama 2013). High-speed centrifugation can also induce the rapid accumulation of host acidosomes and lysosomes at the anterior end of *Paramecium*. Within 10 min of centrifugation, the

accumulated vesicles recover their original positions by host rapid cytoplasmic streaming (Kodama 2013). This observation showed that although acidosomes and lysosomes were dislocated by centrifugation, the mitochondria remained unchanged. This is probably because acidosomes and lysosomes undergo cytoplasmic streaming, but mitochondria do not.

Figure 5 shows a schematic representation of the algal attachment mechanism beneath the host cell cortex after dislocation via high-speed centrifugation. After the high-speed centrifugation, almost all trichocysts are released out of the cell. The discharged trichocysts were regenerate from the endoplasmic reticulum, pass through the Golgi apparatus, and undergo a maturation process (pretrichocysts) before mature trichocysts are delivered to the cell membrane (Plattner 2017). Pretrichocysts contain a growing mass of electron dense secretory material. The pretrichocysts then elongate while their luminal space is progressively filled with crystallizing secretory materials (Garreau De Loubresse 1993). Because several hours are required for regeneration after trichocyst discharge (Harumoto 2002), no trichocysts are observed in the cells after algal reattachment. This representation can be defined by obtaining monoclonal antibodies against both mitochondria and trichocysts. To the best of our knowledge, this is the first report showing the distribution of host mitochondria and trichocysts during algal relocalization beneath the host cell cortex.

Fast cytoplasmic streaming after high-speed centrifugation of *P. bursaria* is the driving force for algal relocalization. The characteristic feature of rotational cytoplasmic streaming in the genus *Paramecium* (*P. aurelia*, *P. caudatum*, *P. bursaria*, *P.*

multimicronucleatum, and *P. calkinsi*) is that the pattern of its route and direction remain constant (Sikora 1981). Various factors, such as cell division, conjugation, temperature, and osmotic pressure, are known to affect cytoplasmic streaming in *Paramecium* (Sikora 1981), but the detachment of the symbiotic algae by high-speed centrifugation is also a factor. In *P. bursaria–Chlorella* endosymbiosis, their cell cycle pace is guaranteed to be synchronous (Takahashi 2016). Takahashi *et al*. (2007) found that *P. bursaria* controls the proliferation of endosymbiotic algae through host-cell-~~,~~ cycle-dependent cytoplasmic streaming. Thus, host cytoplasmic streaming plays a major role in maintaining the number and cellular localization of symbiotic algae in *P. bursaria* cells.

The mitochondria of the host *P. bursaria* are close to the PV membrane (Fig. 1), and their number and function are altered by the presence or absence of symbiotic algae (Kodama and Fujishima 2022). Therefore, host mitochondria and symbiotic algae are expected to be strongly associated with each other. We assumed that the presence of mitochondria was essential for the existence of symbiotic algae; however, the results in Fig. 4 contradict this expectation. Mitochondria and endoplasmic reticulum (ER) are contact at ER-Mitochondria Contact Sites (EMCS)s. Lipids and Ca^{2+} synthesized in the ER are transported to mitochondria through the EMCSs (Kornmann *et al*. 2009; Rizzuto *et al*. 1998). Furthermore, EMCs is also important in reactive oxygen species (ROS) production (Booth *et al*. 2016) and autophagosome formation (Hamasaki *et al*. 2013). Song *et al.* (2017) reported that the host ~~endoplasmic reticulum~~ER is also involved in organizing intracellular algal symbiosis in the cytoplasm. The relationship between host

mitochondria and symbiotic algae and the relationship between other cellular organelles, including the host endoplasmic reticulum, should be examined in detail in future studies.

The exposure of *P. bursaria* cells to ultraviolet and photosynthetically active radiation is known to induce symbiotic algal dislocation, moving to the posterior cell region (Summerer *et al.* 2009). The displaced algae relocate to a "normal" distribution when host cells are transferred to a medium without ultraviolet radiation conditions. The mechanisms of algal displacement and relocation remain unclear (Summerer *et al.* 2009). A dislocation of *Chlorella* symbionts has also been described in the ciliate *Pelagodileptus trachelioides* (Butkay 2004). Although this phenomenon appears to be a stress reaction followed by host cytolysis, the details remain unknown. The method of artificial algal detachment and subsequent rapid reattachment by high-speed centrifugation of algae-bearing *P. bursaria* may contribute to understanding the mechanism of symbiotic algal localization in a variety of host species.

Although the mechanism of PV localization beneath the host cell cortex is still unknown, the docking or distribution mechanism of mitochondria and uninserted trichocysts in other ciliate species, such as *Paramecium tetraurelia,* have been reported. Aufderheide (1977) reported that, in the subcortical regions of *P. tetraurelia*, mitochondria and uninserted (undocked) trichocysts display saltatory motility with individual characteristics, making them distinguishable from each other and from cellular cyclosis. The saltatory motion of trichocysts is implicated as a means of transporting new trichocysts from the cytoplasm to their ultimate location in the cellular

cortex. In addition, the saltatory motion may be a factor in the intracellular distribution of the mitochondria. The mechanism of trichocyst docking at the *Paramecium* cell membrane has been reported in detail (Plattner 2017; Plattner *et al.* 1982). Before trichocyst docking, mature trichocysts move through the subcortical region by cyclosis (Sikora 1981). Then, they approach, in a saltatory manner, tip first, a docking site at the cell membrane (Aufderheide 1978); Thereby, trichocysts are guided by the same "hand." This entity is a microtubule emanating from a nearby ciliary basal body (Plattner *et al.* 1982), which always is situated ~ 0.5 to 1 μm from a docking site proper. Petrou *et al.* (2017) found that the intracellular algal symbionts within the large photosymbiotic foraminifera *Marginopora vertebralis* exhibit phototactic behavior and the phototactic movement of the symbionts is accomplished by the host through rapid actin-mediated relocation of the symbionts deeper into the cavities within the calcite skeletons. Thus, the host cytoskeleton plays a major role in the migration of host cytosolic organelles and symbiotic algae. In order to study the involvement of the cytoskeleton in the process of PV localization, the cell cortex of algae-bearing *P. bursaria* cells was observed in detail by TEM. However, no cytoskeletal structure that seems to be related to PV localization has been found until now (Kodama unpub. Data).

Adhesion just below the host cell surface is a universal phenomenon found not only in cell organelles and symbiotic algae but also in pathogenic bacteria. Protists are potential natural hosts of various bacterial species, including pathogens. *Paramecium caudatum* could be a natural reservoir of *Legionella pneumophila* (Watanabe *et al.* 2016), and *P. bursaria* could be a potential host of *Francisella novicida* (Watanabe *et*

al. 2022). *Francisella novicida* is a facultative intracellular pathogen and a causative agent of tularemia (Ellis *et al.* 2002). Recently, Watanabe *et al.* (2022) found that *F. novicida* (strain U112) cells, which were wrapped with the host DV membrane, localized beneath the host cell cortex. This indicates that both symbiotic algae and bacteria can attach beneath the host cell cortex. Therefore, elucidation of the mechanism of localization beneath the host cell cortex using *P. bursaria* is expected to contribute to the development of not only symbiotic biology but also infectious disease research.

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Consent to participate

All authors have their consent to participate.

Consent to publish

All authors have their consent to publish their work.

Contributions

Conceived and designed the experiments: YK and MF. Performed the experiments: YK.

Wrote the paper: YK and MF.

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Not applicable.

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