Review

# Four important cytological events needed to establish endosymbiosis of symbiotic *Chlorella* sp. to the alga-free *Paramecium bursaria*

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## SUMMARY

Each symbiotic *Chlorella* of the ciliate *Paramecium bursaria* is enclosed in a perialgal vacuole (PV) derived from the host digestive vacuole (DV) to protect from lysosomal fusion. Irrespective of the mutual relationships between *P. bursaria* and their symbiotic algae, the alga-free paramecia and symbiotic algae still have an ability to grow independently and can be experimentally reinfected by mixing them. This phenomenon provides an excellent opportunity to elucidate cell-to-cell interactions between protozoa and algae during establishment of the secondary endosymbiosis. However, the detailed algal reinfection process had been unclear. Therefore, using pulse-label of the alga-free paramecia with the isolated symbiotic algae and chase method, we found four important cytological events needed to establish endosymbiosis. (1) 3 min after mixing, some algae show resistance to the host lysosomal enzymes in the DVs even if the digested ones are coexisted. (2) 30 min after mixing, the alga starts to leave from the DV to appear in the cytoplasm by budding of the DV membrane. (3) Within 15 min after the algal appearance in the cytoplasm, the vacuole enclosing a single green alga differentiates into the PV from the DV, which gives protection from the host lysosomal fusion. (4) After that, the alga localizes beneath the host cell cortex. At about 24 h after mixing, the alga increases by cell division and establishes endosymbiosis. In this review article, we describe our recent studies on the four events during the algal reinfection process.

Key words: *Chlorella* spp., Digestive vacuole, Endosymbiosis, *Paramecium bursaria*, Perialgal vacuole, Reinfection

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## INTRODUCTION

Endosymbiosis acts as a primary force for the biodiversity. Recent studies on algal evolution have shown that endosymbiosis has occurred several times and has brought a diversity of eukaryotic cells. Despite the importance of this phenomenon, molecular mechanisms of the establishment of endosymbiosis between eukaryotic cells are still not well known.

Ciliate *Paramecium bursaria* cells harbor several hundreds of symbiotic *Chlorella* spp. in their cytoplasm (Fig. 1). Each symbiotic alga is enclosed in a special membrane called perialgal vacuole (PV) membrane derived from the host digestive vacuole (DV) membrane (Fig. 2). PV membrane has a function to protect the symbiotic alga from host lysosomal fusion (Gu et al., 2002; Karakashian and Rudzinska, 1981; Kodama and Fujishima, 2009b).

Various benefits are induced in both the host *P. bursaria* and the algae by algal infection. For the symbiotic algae, the host can supply the algae with nitrogen components and $CO_2$ (Albers and Wiessner, 1985; Albers et al., 1982; Reisser, 1976; Reisser, 1980). The algae are protected from infection of the *Chlorella* virus when the alga is wrapped with the PV membrane (Kawakami and Kawakami, 1978; Reisser et al., 1988; Van Etten et al., 1991; Yamada et al., 2006). Algal carbon dioxide fixation is enhanced by the host extracts. Recently, the active substances are identified as three cations ($Ca^{2+}$, $K^{+}$, and $Mg^{2+}$) (Kamako and Imamura, 2006; Kato and Imamura, 2009). On the other hand, for the host paramecia, the algae can supply the host with a photosynthetic product, mainly maltose (Brown and Nielsen, 1974; Reisser, 1976, 1986). Inside the host cell, the algae show a higher rate of photosynthetic oxygen production than in the isolated condition, thereby guaranteeing an oxygen supply for their host (Reisser, 1980). Recently, Kato and Imamura (2009) updated a schematic representation of materials that are supposedly transported, and metabolic controls between the host and the symbiotic algae. Furthermore, alga -bearing *P. bursaria* cells can divide better than

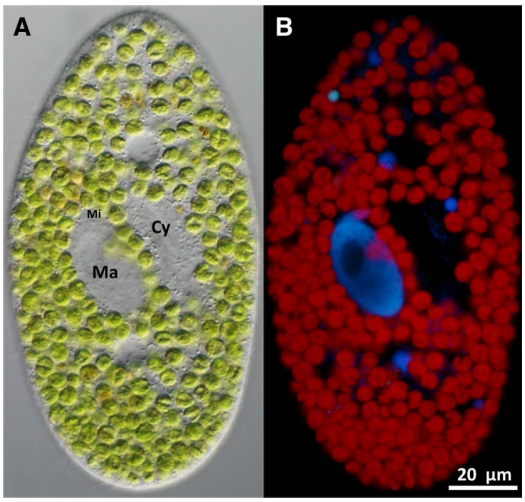


Fig. 1. Light (A) and fluorescence (B) micrographs of alga -bearing *P. bursaria*. Hundreds of symbiotic *Chlorella* sp. are observed in the *Paramecium* cytoplasm (A). Fluorescence of chlorophyll within chloroplast is red color. Macronucleus (Ma) and micronucleus (Mi) are stained with DAPI (blue) (B). Cy, cytopharynx. From Kodama and Fujishima (2010c).

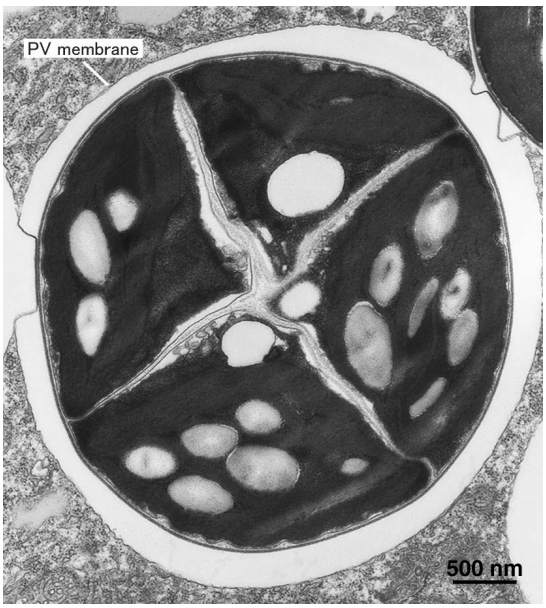


Fig. 2. Transmission electron micrograph of PV membrane enclosing dividing symbiotic alga. From Kodama and Fujishima (2010c).

the alga-free cells (Görtz, 1982; Karakashian, 1963, 1975). Alga-bearing cells show a higher survival rate under various stressful conditions than the alga-free cells. For example, under 0.5 mM nickel chloride ($NiCl_2$), or at high temperatures (42°C), or in 150 mM hydrogen peroxide ($H_2O_2$) (Kinoshita et al., 2009; Miwa, 2009). Recently, Summerer et al. (2009) found that the host paramecia can receive protection against UV damage by their symbiotic algae. Furthermore, because the timing of the cell divisions of both algae and the host paramecia is well coordinated, the symbiotic algae can be distributed to the daughter cells (Kadono et al., 2004; Takahashi et al., 2007).

As described above, though the relationship between *P. bursaria* and *Chlorella* spp. is a mutualism, the alga-free cells and the symbiotic algae are still keeping an ability to grow without a partner. Alga-removed *P. bursaria* cells can be produced easily from alga-bearing cells using one of the following methods: rapid cell division (Jennings, 1938); cultivation under the constant dark condition (Karakashian, 1963; Pado, 1965; Weis, 1969); X-ray irradiation (Wichterman, 1948); treatment with 3-(3,4-dichlorophenyl)-1,1-dimethylurea (DCMU), a blocker of electron flow in photosystem II (Reisser, 1976); treatment with the herbicide paraquat (Hosoya et al., 1995) or treatment with a eukaryotic protein synthesis inhibitor, cycloheximide (Kodama and Fujishima, 2008; Kodama et al., 2007, 2011; Weis, 1984). On the other hand, symbiotic algae can be isolated from alga-bearing *P. bursaria* cells by sonication, homogenization (Kodama and Fujishima, 2005), or treatment with some detergents (M. Yasuda and M. Fujishima, unpublished data). Furthermore, by mixing the alga-removed *P. bursaria* and the isolated symbiotic algae, endosymbiosis can be easily established again (Karakashian, 1975; Siegel and Karakashian, 1959; Kodama and Fujishima, 2005). In addition to these features, *P. bursaria* have some usefulness as follows: *P. bursaria* can be cultivated easily and can get mass culture; the reinfection process of the algae can be observed easily under a light microscope; an alga-free mutant strain of *P. bursaria* has been collected (Tonooka and Watanabe, 2002, 2007). For these reasons, *P. bursaria* and their symbiotic *Chlorella* spp. are considered an excellent model for studying cell-to-cell interaction and the biodiversity through the secondary endosymbiosis. However, the algal infection process to the alga-free *P. bursaria* had remained unknown for a long time. Recently, by using pulse label and chase method, we clarified the algal reinfection process and found four important cytological events needed for establishment of endosymbiosis and their timings during the algal infection process (Kodama and Fujishima, 2005, 2007, 2008, 2009a, 2009b, 2009c, 2010a, 2010b, 2010c, 2010d; Kodama et al., 2007, 2011).

## DIFFERENTIATION STAGE OF DVS OF *P. BURSARIA*

Infection of algae to the alga-free *P. bursaria* is conducted through the host's phagocytosis. To investigate the algal infection process, we first classified the differentiation stages of DVs in phagocytosis by using pulse label and chase method (Kodama and Fujishima, 2005).

### *Pulse label and chase method*

Cells from a 60 ml culture of alga-free *P. bursaria* strain Yad1w or OS1w at the early stationary phase of growth were strained through eight layers of fine gauze, washed twice with modified Dryl's solution (MDS) (Dryl, 1959) ($KH_2PO_4$ was used instead of $NaH_2PO_4 \cdot 2H_2O$), by hand-operated centrifugation, and suspended in 1 ml of MDS. The cells were mixed at a density of $5 \times 10^3$ paramecia/ml with *Chlorella* sp. isolated from the alga-bearing *P. bursaria* cells at $5 \times 10^7$ algae/ml in a centrifuge tube (volume, 10 ml) under a fluorescent light (20–30 μmol photon/$m^2$s) for 1.5 min at 25 ± 1°C. The paramecia-algae mixture was

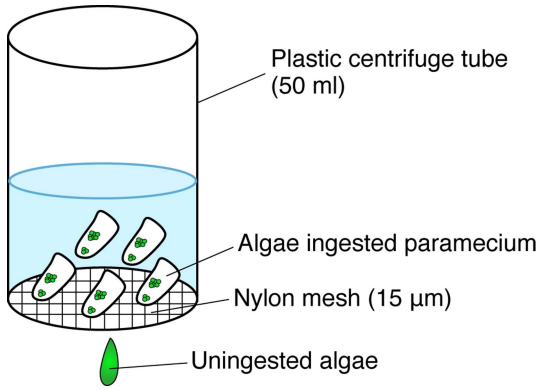


Fig. 3. A centrifuge tube with nylon mesh for removal of algae from *Paramecium-Chlorella* mixture. By pouring 30 ml of MDS, the paramecia are washed, and algae are simultaneously removed through the nylon mesh.

transferred to a centrifuge tube equipped with a 15 μm pore size nylon mesh (Fig.3) and filtered. By pouring 30 ml of fresh MDS into this tube, the paramecia were washed and algal cells outside the paramecia were simultaneously removed through the mesh. The paramecia retained on the mesh were harvested and transferred to a centrifuge tube (volume, 10 ml) and resuspended in 1 ml of MDS, and then chased for various times under a fluorescent light at 25 ± 1°C. Then the cells were observed with or without fixation under a differential interference contrast (DIC) microscope. In a separate procedure, the paramecia-algae mixture was fixed without washing with MDS.

***The DV stages of P. bursaria***

To classify the DV stages of *P. bursaria* that appear during the algal infection process and to determine the timing of the appearance of each stage, algae-containing *P. bursaria*'s DVs were observed under a DIC microscope. The DVs containing several algae were classified into four stages based on their morphological features and color changes of the algae accompanied by algal digestion. The first stage, DV-I is characterized by its round shape and its visible vacuolar membrane. The color of the algae is green. The second stage, DV-II is characterized by its condensed vacuolar membrane. The color of the algae is still green. The third stage, DV-III is characterized by its round shape and its visible vacuolar membrane like DV-I. In this stage, the color of the some algae becomes faintly yellow accompanied by their digestion. Color of the undigested alga is green. So, we classified DV-III into three substages. DV-IIIa contains undigested green algae only. DV-IIIb contains both digested yellow and undigested green algae. DV-IIIc contains digested yellow algae only. In the final stage, DV-IV is characterized by its condensed vacuolar membrane like DV-II. In this stage, the color of digested algae becomes brown and algal diameter becomes small. So, we also classified DV-IV into three substages. DV-IVa contains undigested green algae only. DV-IVb contains both undigested green and digested brown algae. DV-IVc contains digested brown algae only. Unlike DV-II, DV-IV was not observed in cells fixed before 3 min, but was observed 20–30 min after mixing. The algae in each DV of nonfixed paramecia showed the same characters in those of fixed paramecia.

To determine the timing of the appearance of each stage of DVs, alga-free *P. bursaria* cells were mixed with isolated symbiotic algae. After that, the paramecia-algae mixture was fixed at every 10 s intervals for 60 s after the mixing. The fixed cells were classified into four stages according to the most advanced stage of DV in the cell (i.e., if both DV-II and DV-III were present in a cell, the cell was classified as a cell with DV-III). As a result, DV-II started to appear in the cells fixed at 30 s after mixing. Furthermore, to determine the timing of the appearance of DV-III, alga-free cells were mixed with isolated symbiotic algae for 1.5 min, washed with MDS, chased, and fixed at every 1 min interval for 10 min after the mixing. As a result, DV-III started to appear in cells fixed at 3 min after mixing.

***Timing of the fusion of acidosomes and lysosomes to the DVs***

In *P. multimicronucleatum*, it has been reported that intravacuolar pH was changed accompanied by acidosomal and lysosomal fusion to the DVs (Fok et al., 1982). To determine the timing of the acidosomal and lysosomal fusion to the *P. bursaria*'s DVs, we examined the change of the intravacuolar pH of *P. bursaria*'s DVs with the use of yeast *Saccharomyces cerevisiae* cells stained with pH indicator dyes. Yeast cells were stained with three kinds of pH indicator dyes, Congo red, bromocresol green (BCG), and bromophenol blue (BPB). Then, the yeast cells were mixed with alga-free *P. bursaria*. Then the color changes of the yeast cells inside the DVs were monitored under a DIC microscope. The color changes of each dyes occurring in phthalic acid and citric acid buffers are confirmed beforehand. These changes did not differ between the buffers, but they were dependent on the pH. Within 0.5 min after mixing, the color of the BCG-labeled yeast cells was blue. This means that the intravacuolar pH was 6.4–7.0. At 1–2 min after mixing, the color of the yeast cells became yellow-green. This means that the intravacuolar pH decreases to 2.4–3.0. Morphological differentiation of DV-II from DV-I also occurred at 0.5–1 min after mixing. These results show that acidosomal fusion to the DV occurs at 0.5–1 min after ingestion. Using Congo-red labeled yeast, identical results were obtained (Kodama and Fujishima, 2005).

Partially digested yellow algae started to appear in DV-IIIb at 2–3 min after mixing. In the late stage of DV-II, the color of the BCG-labeled yeast cells became blue, indicated that the intravacuolar pH was already 6.4–7.0. These results suggest that lysosomal fusion might start before 2–3 min after mixing. Gomori's staining (Gomori, 1952), which is used to detect acid phosphatase (AcPase) activity in the DVs showed that both DV-I and DV-II are AcPase-activity negative, but all substages of DV-III and DV-IV are AcPase-activity positive (Kodama and Fujishima, 2009a) (see *Timing of the PV membrane differentiation* in this review). These morphological and histochemical results show that the lysosomal fusion occurs before 2–3 min after mixing. A schematic representation of DV differentiation of *P. bursaria* is depicted in Fig.4.

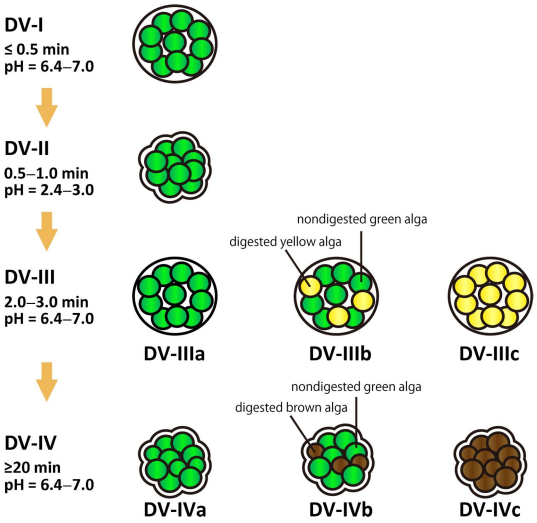


Fig. 4. Schematic representation of DV stages of *P. bursaria*. When *Chlorella* sp. and alga-free paramecia are mixed, one or several algae pass through the host cytopharynx and are pinched off as DV-I. The pH inside DV-I is 6.4–7.0. Condensed DV-II appears 0.5–1.0 min after mixing. The pH inside DV-II reduces to 2.4–3.0. Lysosomal fusion occurs at 2.0–3.0 min, leading to swollen DV-IIIa to DV-IIIc. Color of some algae becomes yellow because of the algal digestion. Digested algae are observed in DV-IIIb and DV-IIIc. The pH inside DV-III increases to 6.4–7.0. Condensed DV-IVa, DV-IVb, and DV-IVc are observed at 20–30 min. The pH inside DV-IV remains at 6.4–7.0. The digested algae inside the DV-IVb and DV-IVc become brown by an additional digestion. From Kodama and Fujishima (2010c).

## FATES OF *CHRORELLA* SP. DURING THE ALGAL REINFECTION PROCESS

As described above, single green *Chlorella* (SGC) is enclosed in a PV membrane as shown in Fig. 2. This fact indicates that timing of the appearance of the SGC can be considered as almost the same as that of the appearance of the PV mem-

brane from DV membrane during the algal infection process. To determine the timing of the appearance of the SGC, alga-free paramecia were pulse labeled with isolated symbiotic algae for 1.5 min, chased, then fixed at 0.05, 0.5, 1, 1.5, 2, 3, 6, 9, 24, 48, and 72 h after mixing. The percentages of paramecia with DV-IIIa or DV-IIIb, DV-IVa or DV-IVb, and SGC were monitored. As a result, all SGCs that were present in the host cytoplasm before 0.5 h after mixing were all digested. However, 1 h after mixing, SGCs, which appeared from either DV-IVa or DV-IVb were observed again in the host cytoplasm. At 24 h, the SGCs began to multiply by cell division (see **INITIATION OF THE ALGAL CELL DIVISION FOR ESTABLISHMENT ENDOSYMBIOSIS** in this review), indicating that these algae could establish endosymbiosis with alga-free *P. bursaria* cells. This result shows that the SGCs to establish endosymbiosis appear from the DV-IVa or the DV-IVb occurs after acidosomal and lysosomal fusion to the DV.

As mentioned above, the SGCs, which could establish endosymbiosis appeared from either DV-IVa or DV-IVb. To clarify the origin of SGCs, the percentages of the paramecia with DV-IVa, DV-IVb, and SGC were monitored by observation of fixed cells at 0.5, 1, 1.5, 2, 3, 6, 9, 24, 48, and 72 h after 1.5-min pulse label with isolated *Chlorella* sp. The percentage of the paramecia with SGC was 0% at 0.5 h and increased as time elapsed and became to 35% at 72 h after mixing. In contrast, the percentage of paramecia with DV-IVb was decreased. On the other hand, less than 5% of cells contained DV-IVa, if any throughout the whole infection process. This indicates that the majority of the SGCs established endosymbiosis with alga-free *P. bursaria* cells originated from DV-IVb (Kodama and Fujishima, 2005).

In contrast to results of an earlier study of Meier and Wiessner (1989), our observations suggest a new escape mechanism from host lysosomal digestion by *Chlorella* sp. during the algal infec-

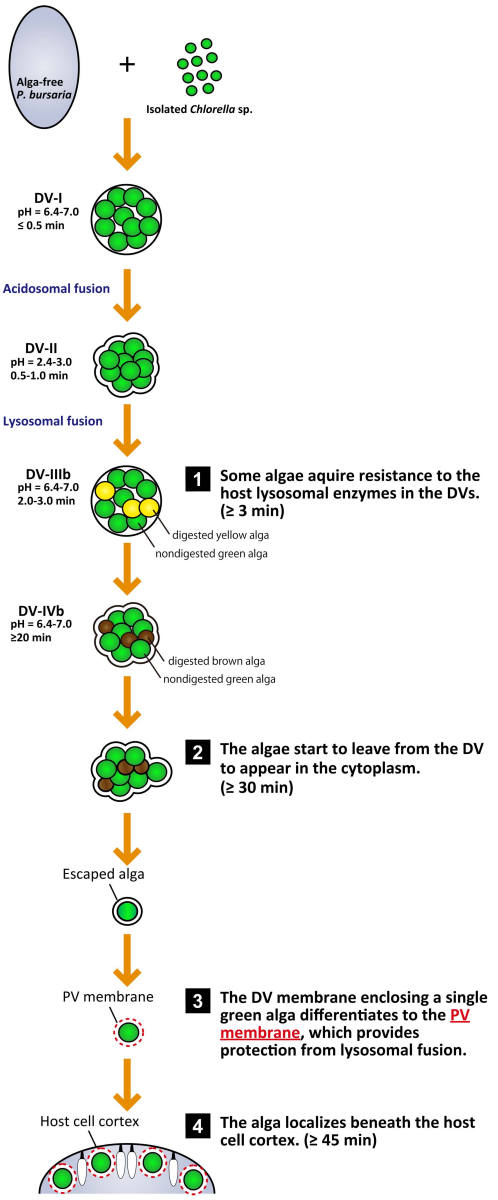


Fig. 5. Algal reinfection process to the alga-free *P. bursaria* and four important cytological events needed to establish endosymbiosis. Using pulse label and chase method, algal reinfection process and four important cytological events necessary to establish endosymbiosis were clarified. First, from 3 min after mixing, some algae acquire temporary resistance to the host lysosomal enzymes in the DVs. Second, from 30 min after mixing, the algae start to leave from the DVs to appear in the host cytoplasm. Third, within 15 min after the algal appearance, the DV membrane enclosing a SGC differentiated into the PV membrane, which provides protection from lysosomal fusion. Finally, the alga localizes beneath the host cell cortex. At about 24 h after mixing, the alga increases by cell division to establish endosymbiosis.

tion process. First, some alga shows temporary resistance to the host lysosomal enzymes in both DV-III and DV-IV. Second, the SGCs to establish endosymbiosis appear from the DV-IVb into the host cytoplasm. Finally, the alga loses their temporary resistance to the host lysosomal enzymes but is protected from lysosomal fusion by wrapping with the PV membrane (Kodama and Fujishima, 2005). Schematic representation of the infection process by *Chlorella* sp. is shown in Fig.5.

## FOUR CYTOLOGICAL EVENTS NEEDED TO ESTABLISH ENDOSYMBIOSIS

During the algal reinfection process, we found four cytological events needed to establish endosymbiosis (Kodama and Fujishima, 2009c, 2010a, 2010c) (Fig. 5). First, from 3 min after mixing, some algae acquire temporary resistance to the host lysosomal enzymes in the DVs, even when the digested ones coexist. Second, from 30 min after mixing, the algae start to leave from the DV to appear in the cytoplasm. Third, within 15 min after the algal appearance, the DV membrane enclosing SGC differentiate into the PV membrane, which provides protection from lysosomal fusion. Finally, the algae localize beneath the host cell cortex. From next section, some results related to each event will be described.

### *Algal resistance to the host lysosomal enzymes in the DVs*

The origin of SGCs that can establish endosymbiosis was DV-IVb, as described above. Approximately 50% of paramecia at 0.5 h after mixing had DV-IVb (Kodama and Fujishima, 2005); most of the DVs at this time were AcPase activity-positive (Kodama and Fujishima, 2009a), which shows that some of the algae in DV-IV acquire resistance to the lysosomal enzyme resistance in the DVs, although the remaining algae are digested in the same DV. Boiled algae are all digested in DV-III when they are added to the alga-free paramecia (Kodama and Fujishima, 2005). Furthermore, all isolated symbiotic algae fixed with 2.5% (w/v) glutaraldehyde or with 5.0% (w/v) formaldehyde are also digested in DV-III (Kodama and Fujishima, 2005). Therefore, it appears that only living algae can show temporary resistance to the lysosomal enzymes in the DVs, which were fused with host lysosomes. To date, the factor determining whether the algal fates survive or are digested in DV-IVb has remained unclear.

*Light microscopy of Chlorella variabilis cells in DV-IVb*

It is known that different types of algae infecting *P. bursaria* clones show different infection ratio and host dependencies (Nakahara et el., 2003; Nishihara et el., 1999). This suggests a possibility that when different *Chlorella* species or strains used together for the infection experiment, it may yield different algal fates in DV-IVb as shown in Fig. 6. To examine this possibility, alga-bearing *P. bursaria* cells strain OS1g1N that was produced by infection of alga-free *P. bursaria* OS1w cells with symbiotic algal clone 1 N cells was used for symbiotic algal isolation (Kodama et al., 2007). This algal clone 1 N was identified as *C. variabilis* (Hoshina et al., 2010) (formerly *C. vulgaris*). Although genetically identical symbiotic algal cells were used for the infection, DV-IVb was appeared (Kodama et al., 2007).

Light microscopy showed that both digested brown and nondigested green algae presented in DV-IVb did not show a specific location, indicating that their different fates are not decided by their positions in the DV. To determine whether the survived algae in the DV-IVb had been wrapped with their original PV membrane, the isolated symbiotic algae were treated with Triton X-100 or Tween-20 detergents before mixing with alga-free *P. bursaria* cells. There were no significant differences in the percentage of the cells with DV-IVb between paramecia mixed with nontreated and detergent-treated algae. Furthermore, we

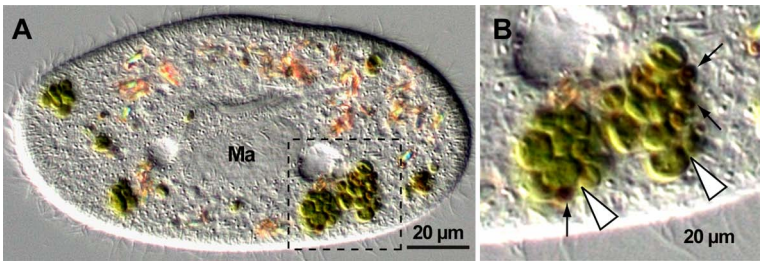


Fig. 6. Photomicrographs of *P. bursaria* 1 h after mixing with algae. B shows a highly magnified image of the square enclosed area in A. As shown by arrowhead in B, some algae were not digested even if they are coexisted with the digested ones (small arrow in B) in the DV-IVb. Ma, macronucleus. From Kodama and Fujishima (2010c).

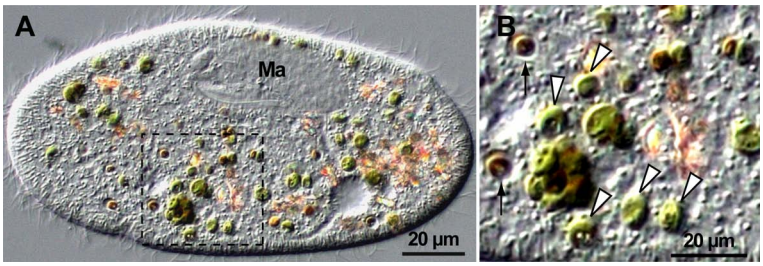


Fig. 7. Photomicrographs of *P. bursaria* 2.5 h after mixing with algae. B shows highly magnified images of the square enclosed area in A. Single green *Chlorella* (SGC, white arrowhead) and single digested *Chlorella* (SDC, black arrow) appear as a result of the budding of the DV-IVb membrane. Note that this algal budding is observed notwithstanding the alga is nondigested or partially digested. Ma, macronucleus. From Kodama and Fujishima (2010c).

found no relationships between algal stage in the cell cycle and their fates in DV-IVb (Kodama et al., 2007).

*Transmission electron microscopy of DV-IVb*

To confirm the possibility that some algae might have acquired a PV membrane in the DV-IVb prior to their budding from the DV-IVb into the host cytoplasm, the DV-IVb was observed by transmission electron microscope (TEM). TEM observation showed that, in the DV-IVb, nondigested algae appear as electron-dense cells and digested algae appear as electron-translucent cells with empty spaces between the cell wall and their cytoplasm. Gu et al. (2002) reported that the space between the PV membrane and the algal cell wall is about 25–100 nm in width. Any membrane resembling a PV membrane was not observed around the nondigested algae. This indicates that the algal fates are not determined by PV membrane acquisition in the host DV-IVb, and that the PV membrane is formed during or after algal budding from the DV-IVb (Kodama et al., 2007).

*Effects of protein synthesis inhibitors, cycloheximide and puromycin on algal survival in DV-IV during infection*

To determine whether algal or host protein synthesis is needed for the survival of the alga in the host DV-IV, isolated symbiotic *C. variabilis* and alga-free *P. bursaria* were pretreated with cycloheximide or puromycin. As a result, most of the paramecia formed DVs enclosing algae 3 min after mixing with or without cycloheximide treatment. The percentage of cycloheximide-treated cells with DV-IVb 3 h after mixing was not significantly different from that of the control experiment.

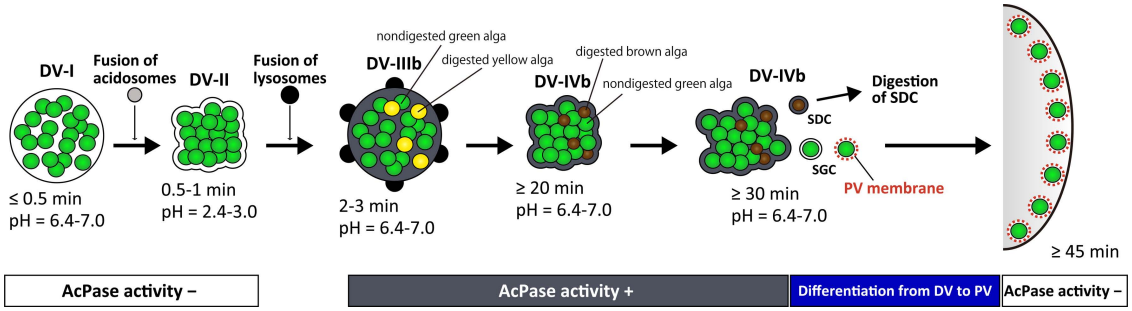


Fig. 8. Schematic diagram of timing of PV membrane differentiation from DV membrane during the algal infection process. AcPase-activity positive area is shown by a gray color in the diagram. Both inside the DV-I and DV-II are AcPase-activity negative. After lysosomal fusion, AcPase-activity appears in DV-III. In the DV-IIIb, some algae show resistance to the lysosomal enzymes and maintain green color and original morphology. Remaining algae are partially digested and show yellow color in the same DV. DV-IIIb differentiates into DV-IVb which containing both nondigested green and digested brown algae. Appearance of the SGCs from DV-IVb begins from 30 min by budding of the DV membrane into the cytoplasm. In the budded membrane, each alga is surrounded by an AcPase activity-positive thin layer. At about 45 min after mixing with algae, the vacuoles enclosing a SGC move quickly and attach just behind the host cell cortex. Such vacuoles are AcPase activity-negative. This indicates that the PV membrane differentiates soon after the appearance of the SGCs from the host DV (updated Kodama and Fujishima, 2009a).

On the other hand, number of formed DVs was significantly reduced to about half by puromycin treatment, although DV-IVb appeared 3 h after mixing. These results show that neither algal nor host protein synthesis are not needed for algal acquisition of lysosomal enzyme resistance in the host DV (Kodama et el., 2007).

### *Algal budding from the host DV membrane*

Some algae start to leave from the DV-IVb 30 min after mixing with the paramecia. This phenomenon can be observed even in cases where the alga was nondigested or partially digested (Fig. 7). Furthermore, this budding was induced not only by living algae, but also by the boiled or fixed algae (Kodama and Fujishima, 2005). Intact or boiled yeast *S. cerevisiae* cells also budded from the DVs (Y. Kodama and M. Fujishima, unpublished data). Inorganic polystyrene latex spheres of 3.0 μm in diameter budded from the DVs, too (Y. Kodama and M. Fujishima, unpublished data). However, this budding was not induced when India ink, 0.81 μm in diameter of polystyrene latex spheres, or food bacteria *Klebsiella pneumoniae* were ingested into the DVs (Kodama and Fujishima, 2005). These observations raise a possibility that *P. bursaria* can recognize the diameter of the content of the DVs, and content with a similar size to algae can bud from the DVs into the cytoplasm. To date, the molecular mechanisms for the budding from the DVs are remained unknown.

### *Differentiation into the PV membrane from the DV membrane*

#### *Timing of the PV membrane differentiation*

To determine the timing of differentiation of the PV membrane from the DV membrane during the algal infection process, alga-free *P. bursaria* cells were mixed with isolated symbiotic algae for 1.5 min, washed with MDS, chased, and fixed at various times after mixing. Then we clarified the timing of the disappearance of AcPase activity during the algal reinfection process using Gomori's staining method because the PV membrane shows no AcPase activity. Fig. 8 shows presence or absence of AcPase activity inside the DV membrane during the algal infection process. AcPase activity started to appear in 2–3-min-old vacuoles, DV-III. Almost all DV-IVs containing algae showed AcPase activity at 30 min. Appear-

ance of the SGCs from the DV-IVb begins at 30 min as described before. In the budded membrane, the alga was surrounded by an AcPase activity-positive thin layer. At about 45 min after mixing, the vacuoles enclosing a SGC moved quickly and became attached beneath the host cell cortex. Inside such vacuoles is AcPase activity-negative. These results show that the PV membrane differentiates soon after the appearance of the SGCs from the host DV and is completed prior to the algal localization beneath the host cell cortex.

*Properties of the PV membrane*

To date, the following qualitative differences are reported between the DV and the PV membrane: 1. The PV membrane encloses only one symbiotic alga (Gu et al., 2002; Karakashian and Rudzinska, 1981); 2. The space between the algal cell wall and the PV membrane is about 0.05 μm, so the PV membrane is hardly observable under a light microscope (Reisser, 1986); 3. The diameter of the PV does not vary much, except during the algal cell division of the enclosed symbiotic alga (Reisser, 1992); 4. The PV does not involve in cytoplasmic streaming, but attaches beneath the host cell cortex (Kodama and Fujishima, 2005; Reisser, 1986); 5. Particle density and its distribution of the PV membrane show few signs hinting at any endocytotic or exocytotic activity (Meier et al., 1984).

*The ability of the PV membrane to protect the alga from host lysosomal fusion*

The timing of differentiation of the PV membrane from the host DV membrane is estimated to occur soon after the algal budding from the host DV membrane and before the localization of the PV membrane beneath the host cell cortex, as described above. However, there was no direct evidence demonstrates that the PV membrane does not allow lysosomal fusion to the membrane. The PVs containing SGC of about 3–4-μm in diameter are embedded between the trichocysts beneath the host cell cortex. We clarified that the cortex area of 5–10-μm depth was AcPase activity-negative by Gomori's staining (Kodama and Fujishima, 2008, 2009a). These observations raise a possibility that the PV membrane does not have an ability to protect the alga from lysosomal fusion, but can avoid lysosomal fusion by localization at the primary lysosome-less area of the host cell cortex. This AcPase activity-negative area could be reduced to less than 3-μm depth at the dorsal surface when the trichocysts are removed by the treatment with lysozyme. In such cells, some of the PVs were exposed to the AcPase activity-positive area. However, such PVs were not stained with Gomori's staining, and the algae in each PV were not digested (Kodama and Fujishima, 2009b). These results demonstrated that the PV membrane does not require the trichocysts for the localization beneath the host cell cortex, and that the PV membrane, unlike the DV membrane, provides protection from host lysosomal fusion, although molecular mechanisms of the PV membrane to give protection from lysosomal fusion are still unknown.

*Synchronous swelling of PVs*

Cycloheximide is known to inhibit the protein synthesis of the symbiotic algae of *P. bursaria* preferentially, but to inhibit host protein synthesis only slightly (Kodama and Fujishima, 2008; Kodama et al., 2007; Weis, 1984). Previously, we found that the PV membrane swells synchronously when the alga-bearing paramecia are treated with cycloheximide for 24 h (Fig. 9A, Kodama and Fujishima, 2008). This phenomenon was tentatively designated as "synchronous PV swelling (SPVS)". The gap between the symbiotic algal cell wall and the PV membrane expanded to about 25 times than its general width after the 24 h treatment. Then, the vacuoles left from beneath the host cell cortex and became stained with Gomori's staining, and the algae in the vacuoles were digested (Fig. 9A, arrow). The SPVS was induced only when the alga-bearing *P. bursaria* are treated with cycloheximide

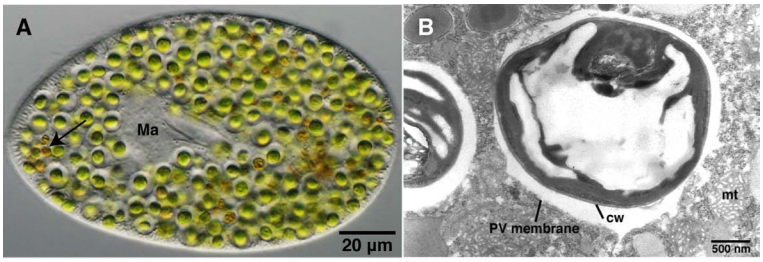


Fig. 9. Light (A) and transmission electron (B) micrographs of cycloheximide-treated alga-bearing *P. bursaria* cells. Twenty-four h after the treatment, all PVs containing green algae swell synchronously (SPVS, Fig. 9A). After the SPVS, some algae start to digest and become brown color by the host lysosomal fusion to the PV membrane (A, arrow). Transmission electron microscopy reveals that the PV membrane expands and the algal ultrastructure is degenerated completely. Ma, macronucleus; cw, algal cell wall; mt, paramecium mitochondrion.

under a constant light (LL) condition, but not under a constant dark (DD) condition. Even under a LL condition, this phenomenon was not induced in alga-bearing *P. bursaria* cells treated with cycloheximide in the presence of the photosynthesis inhibitor DCMU. These results indicate that the algal proteins synthesized in the presence of the algal photosynthesis have some important function to prevent expansion of the PV and to maintain the ability of the PV membrane for localization beneath the host cell cortex and for protection from host lysosomal digestion (Kodama and Fujishima, 2008).

The ultrastructure of the alga inside the swelled PV membrane was observed using TEM. Before cycloheximide treatment, the color of the symbiotic algae was green, and the PV membrane was hardly observable under a DIC microscope. Using TEM, a rippled PV membrane could be observed and a chloroplast with a pyrenoid and other ultrastructural components of the alga were clearly visible. The space between the PV membrane and the algal cell wall was very narrow. At 15 h after treatment, observation by DIC revealed that morphological differences were not occurred in the symbiotic algae, and the PV membrane was not observable. At the same time, though TEM observation showed that most of the PV membrane is located very closely to the alga, part of the PV membrane had started to swell. Furthermore, chloroplasts and other ultrastructural components including the algal nucleus had been completely destroyed. At the same time, many small vesicles appeared around the PV membrane. Although these small vesicles have a similar size to that of lysosomes, these were electron-translucent, unlike the lysosomes reported by Fok et al (1979). Fusion of these small vesicles to the PV membrane was not observed. Ultrastructural changes showing degradation of mitochondria or trichocysts of the paramecia were not observed by treatment with cycloheximide. At 24 h after treatment, the PV membrane started to swell, so the PV membrane were easily visible by DIC microscope. Simultaneously, the algae were started to digest and the color of the algae changed to brown. As shown in Fig. 9B, observation using TEM revealed a wide space between the PV membrane and the algal cell wall and the degeneration of the algal ultrastructure. By 48 h after treatment, the PV membrane had contracted again, and became hardly visible by DIC microscope. Observation using TEM also showed that the PV membrane was contracted again by unknown mechanism (Kodama et al., 2011).

To examine the timing of the onset of the degeneration of the algal ultrastructure, alga-bearing cells treated with cycloheximide were fixed for TEM observation at 1, 3, 6, and 9 h after the treatment under the LL condition. At 1, 3, and 6 h after the treatment, no morphological change

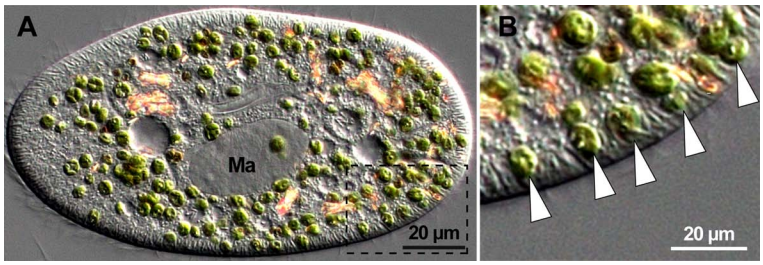


Fig. 10. Photomicrographs of alga-free *P. bursaria* 4 h after mixing with algae. B shows highly magnified image of the square enclosed area in A. As shown by arrowheads in B, SGCs, which were wrapped with PV membrane translocate beneath the host cell cortex and are embedded among the trichocysts. Ma, macronucleus. From Kodama and Fujishima (2010c).

was observed in algal ultrastructure. However, at 9 h, the ultrastructure in many algae had already degenerated. These observations suggest that algal ultrastructure begins to degenerate between 6 h and 9 h after the treatment (Kodama et al., 2011).

The SPVS and subsequent algal digestion induced by treatment with cycloheximide under the LL conditions were also induced in other strains of alga-bearing *P. bursaria* cells: SKS-4-5, HG1g, HG5g, HG18g, and HG24g. These results show that SPVS and subsequent algal digestion by the treatment with cycloheximide are general phenomena that occur in various strains of alga-bearing *P. bursaria* cells (Kodama et al., 2011).

*Effects of other protein synthesis inhibitors, chloramphenicol, emetin, and puromycin on the PV membrane*

Effects of chloramphenicol, an inhibitor of bacterial, chloroplastic, and mitochondrial protein synthesis, and effects of emetine and puromycin, inhibitors of the paramecia's protein synthesis on the SPVS and subsequent algal digestion were examined. Firstly, to determine appropriate concentrations of these inhibitors, effects of these inhibitors on the growth rate of the paramecia were examined. In the case of *P. caudatum*, 3 µg/ml of emetine completely inhibited their cell division (Dohra and Fujishima, 1999). In the case of *P. bursaria*, 0.1, and 0.25 mg/ml of chloramphenicol, 1 µg/ml of emetine, and 60 µg/ml of puromycin inhibited their cell division. However, 0.5% ethanol, which concentration corresponded to that of a solvent of 0.25 mg/ml of chloramphenicol did not inhibit. The effects of these inhibitors on the SPVS and following algal digestion were observed using DIC microscope. All concentrations of chloramphenicol, emetin, or puromycin did not induce both SPVS and subsequent algal digestion, irrespective of LL and DD conditions. These results show that, among the protein synthesis inhibitors examined in our study, only cycloheximide induces SPVS and subsequent algal digestion (Kodama and Fujishima, 2008).

To date, presence of the PV are reported in endosymbiosis between *P. bursaria*, *Climacostomum virens*, *Stentor polymorphus*, *Vorticella* sp., *Spongilla fluviatilis*, and *Hydra viridis* with *Chlorella* sp. (Graham and Graham, 1978, 1980; Karakashian et al., 1968; Oschman, 1967; Reisser, 1981; Reisser et al., 1984). Notwithstanding the importance and generality of the PV membrane, little is known about qualitative characteristics of the PV membrane. Identification of SPVS will be helpful to clarify the PV membrane properties.

***Algal translocation and attachment beneath the host cell cortex***

The SGCs, which appeared from DV-IVb by budding of the DV membrane translocate beneath the host cell cortex and are embedded among the trichocysts as shown in Fig. 10. This phenomenon

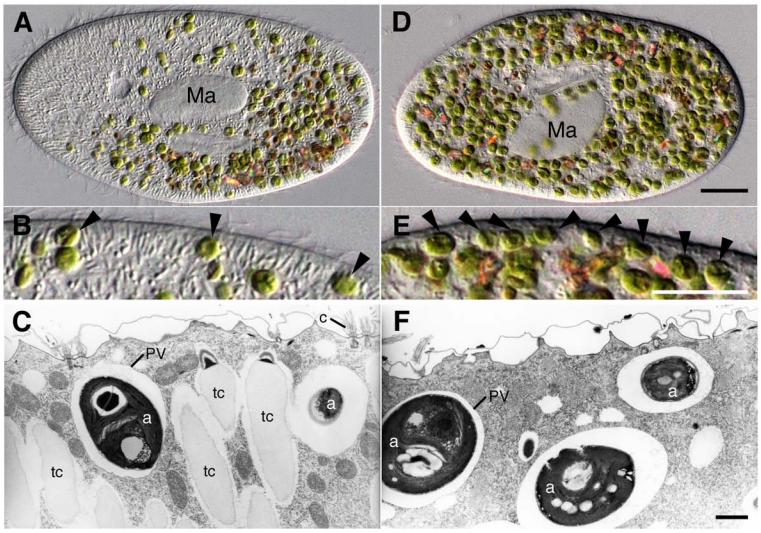


Fig. 11. Light and transmission electron photomicrographs of alga-free trichocysts-removed (D)–(F) or nonremoved (A)–(C) *P. bursaria* cells that were pulse labeled with symbiotic algae. B and E show dorsal surfaces of A and D, respectively. A, B, D and E show DIC images of living paramecia at 3 h after mixing with the algae. C and F show TEM images of paramecia at 3 h after mixing. In a trichocysts-nonremoved cell, the algae move trichocysts aside (A)–(C). Note that the alga can attach beneath the host cell cortex of the trichocysts-removed cell (D)–(F). Arrowheads in B and E show symbiotic algae localized close to the host cell cortex. Ma, macronucleus; a, symbiotic alga localized beneath the host cell cortex; PV, perialgal vacuole membrane; tc, trichocyst; c, cilia; Bars, 20 μm (D and E) and 1 μm (F). From Kodama and Fujishima (2009b).

is observed only with living algae but not with boiled algae (Kodama and Fujishima, 2005). Localization of symbiotic algae has been observed in various protists (Reisser, 1986). These facts indicate that the PV membrane functions not only for protection of the symbiont from lysosomal fusion, but also for stable attachment of the symbiont near the host cell cortex. Tonooka and Watanabe (2002, 2007) reported of a natural alga-free strain of *P. bursaria.* Infection experiments using this strain revealed that this strain couldn't maintain stable endosymbiosis with algae. The algae ingested by the paramecia aggregated at the posterior region of the cell, and then alga-free paramecium was produced after cell division. Crossbreeding analyses between this strain and a normal strain showed that all F1 progenies could establish stable endosymbiosis with the algae, but some F2 progenies, through sibling crosses between symbiotic F1 progenies could not. Their results indicate that the translocation and attachment of the PVs beneath the host cell cortex is genetically controlled by unknown host factor.

Thousands of trichocysts are embedded under the *Paramecium* cell cortex, as defensive organelles against predators (Harumoto and Miyake, 1991). Using lysozyme, discharge of trichocysts in *Paramecium* can be induced without seriously injuring it (Harumoto and Miyake, 1991). Because the symbiotic algae seem to push the trichocysts aside to become localized beneath the host cell cortex after appearance from the host DV, we examined the necessity of trichocysts for the algal localization. To examine the possibility, alga-free paramecia and isolated symbiotic algae were mixed for 1.5 min, washed with MDS, and then resuspended with the same concentration in MDS. Because the SGCs start to appear from DVs at 0.5 h after mixing with algae (Kodama and Fujishima, 2005, 2009b), the pulse-labeled cells were treated with 1 mg/ml lysozyme 0.5 h after mixing. This concentration of lysozyme could induce full dis-

charge of trichocysts of alga-free *P. bursaria* cells. These paramecia were fixed at 3 and 24 h after mixing with algae in the presence of lysozyme, and observed to determine whether the SGCs that had appeared from the DV-IV were able to attach beneath the host cell cortex of the trichocyst-removed paramecia. At 3 h, in the control experiment, SGCs attached beneath the host cell cortex were observed (Figs. 11A–C). These algae looked to move the trichocysts aside. On the other hand, SGCs could localize beneath the host cell cortex, even in cases where the trichocysts had been removed by the treatment of lysozyme (Figs. 11D-F). Interestingly, in the trichocysts-removed cells, the SGCs localized beneath the host cell cortex were more numerous than in the non-removed cells (Figs. 11D and F). With respect to the percentage of the trichocysts-bearing cells with SGCs localized beneath the host cell cortex was about 65%. However, it increased to about 95% in the trichocyst-removed cells by 24 h after mixing. These results demonstrate that the PV membrane does not require the trichocysts for the algal intracellular localization (Kodama and Fujishima, 2010a).

Besides our results, Omura and Suzaki (2003) reported that the density of trichocysts in the alga-bearing *P. bursaria* cells was less than that in the alga-free cells. These observations raise a possibility that the symbiotic algae and the host trichocysts compete for the host cell cortex to attach themselves there. To examine the possibility, we developed a monoclonal antibody (mAb) against the trichocysts and observed the change of trichocysts caused by the algal reinfection to the alga-free cells. Indirect immunofluorescence microscopy shows that an epitope for the mAb localizes in both trichocysts before (Fig. 12A) and after (Fig. 12B) discharge.

The changes in the trichocysts during the algal reinfection process were observed using indirect immunofluorescence microscopy. Three min after mixing with algae, *Paramecium* cells had

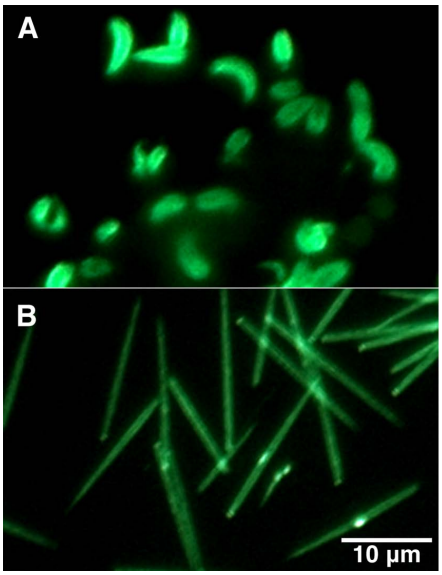


Fig. 12. Indirect immunofluorescence micrographs of *Paramecium* trichocysts with a monoclonal antibody against the trichocysts. Immunofluorescence shows that an epitope for the monoclonal antibody localizes in both trichocysts before (A) and after (B) discharge.

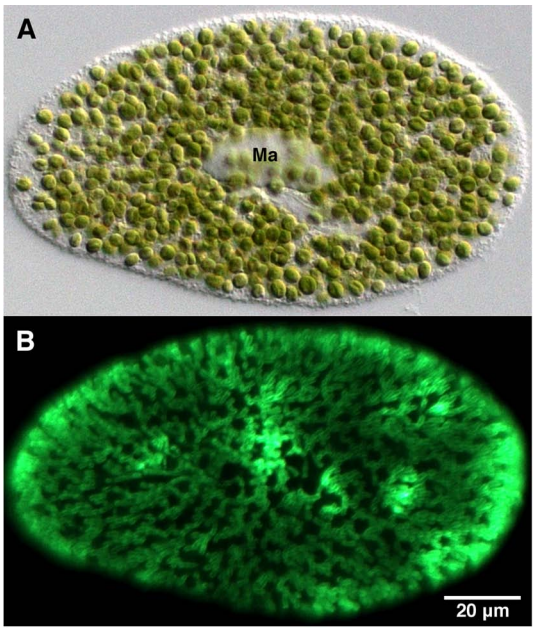


Fig. 13. Indirect immunofluorescence micrograph of alga-bearing *P. bursaria*. A shows a DIC micrograph. B shows an indirect immunofluorescence micrograph. Immunofluorescence shows that the trichocysts are not present in the area where the symbiotic alga localizes. Ma, macronucleus.

many trichocysts under the cell cortex. Three h after mixing, the alga localized beneath the host cell cortex after escaping from the host DVs. At that time, the trichocysts changed their arrangement to make a space for the algae and form a ring surrounding each alga localized beneath the host cell cortex. At about 24 h after mixing, the number of the algae located beneath the host cell cortex has increased (Fig. 13A), because the algae started cell division. The ring-like arrangement of trichocysts increased according to the increase in the number of algae as shown in Fig. 13B. These results indicate that the symbiotic algae compete for their attachment sites with preexisting trichocysts and the algae have the ability to ensure algal attachment sites beneath the host cell cortex.

The changes in the trichocysts when the boiled algae were mixed with alga-free *P. bursaria* cells were also examined. When the boiled algae were mixed with alga-free cells, the algae were ingested into the host DVs just as living ones had been, but all boiled algae in the DVs began to be digested immediately, and the color of the digested algae became yellow. Boiled algae also budded from the DV-IV, though they failed to localize beneath the host cell cortex. The all ingested boiled algae were digested and exocytized from the cytoproct within 72 h after mixing (Kodama and Fujishima, 2005). It is noteworthy that the change of the trichocysts was not observed when the boiled algae were ingested into the alga-free *P. bursaria* cells (Kodama and Fujishima, 2010b). Therefore, the ring-shaped rearrangement of trichocysts is a phenomenon induced by the algal localization beneath the host cell cortex.

Furthermore, the changes in the trichocysts when the living symbiotic algae were mixed with *P. caudatum* cells were examined. When living symbiotic algae isolated from alga-bearing *P. bursaria* cells were added to *P. caudatum* cells, the algae were ingested into the DVs. Within 6 h after mixing, all algae were digested and the color of the algae became brown. Finally, all digested algae were discharged from a cytoproct. During this process, no change was observed in the trichocyst's arrangement. These results show that arrangement of the trichocysts in a ring-like shape is only induced when the living symbiotic algae were ingested by alga-free *P. bursaria* (Kodama and Fujishima, 2010b). Results of these observations showed that symbiotic algae and the *P. bursaria*'s trichocysts are competing for attachment to the host's unknown structures beneath the host cell cortex.

Because trichocysts are attached at predictable sites of the cell cortex to prevent the dislocation (Hausmann, 1978), the trichocysts may not be simply pushed away by the attached algae. Using Gomori's staining method, we clarified that part of the trichocysts beneath the host cell cortex are digested by host lysosomal fusion to reserve the algal attachment sites during the algal reinfection process (Kodama and Fujishima, 2010b). Although, the mechanism of replacement of trichocysts by infection of the symbiotic algae remains unclear, we proposed a hypothesis that trichocysts are not simply pushed away by SGCs, but the trichocysts close to the putative algal attachment site were selectively digested by an unknown mechanism to ensure the algal attachment sites beneath the host cell cortex during algal infection process (Kodama and Fujishima, 2010b).

## INITIATION OF THE ALGAL CELL DIVISION FOR ESTABLISHMENT ENDOSYMBIOSIS

The timing of the initiation of the algal cell division during the algal infection process had been remained unknown for a long time. Therefore, alga-free *P. bursaria* cells at the stationary phase of growth were pulse-labeled with isolated symbiotic algae for 1.5 min, washed with MDS, chased, and fixed at 1, 3, 6, 9, 24, and 48 h after mixing. Then, the mean number of green algae per a *P. bursaria* cell was counted (Kodama and Fujishima, 2005). At 1 h, the mean number of green

algae was 9.4 algae/cell. It decreased to 4.0 algae/cell at 3 h, and remained constant until 9 h. However, the algae began to increase to 5.3 algae/cell at 24 h. In addition, dividing algae were frequently observed at 24 h under a DIC microscopy. Thus, algal cell division initiates at about 24 h after mixing with the alga-free *P. bursaria* cells for establishment endosymbiosis (Kodama and Fujishima, 2005).

## CONCLUSION

More than fifty years ago, Siegel and Karakashian (1959) reported that *P. bursaria* and their symbiotic *Chlorella* can separate and then grow independently, and that reinfection of *Chlorella*-free paramecia is inducible. Since that time, *P. bursaria* and its symbiotic *Chlorella* spp. have been considered as a model for studying endosymbiosis by many researchers. Nevertheless, the detailed algal infection process has not been revealed over those many years. Recently, through pulse-labeling with isolated symbiotic algae from alga-bearing *P. bursaria* for 1.5 min and chasing for various times by our study (Kodama and Fujishima, 2005), classification of *P. bursaria*'s DVs, timing of acidosomal and lysosomal fusion to the DVs, the route of algal infection to the alga-free *P. bursaria* cells, and four important cytological events needed to establish endosymbiosis were found as summarized in Fig. 5.

To establish endosymbiosis with the host, an endosymbiont has to meet the following requirements: (1) An endosymbiont invades the host cytoplasm. (2) The endosymbiont avoids digestion by the host's lysosomal fusion. (3) The endosymbiont grows synchronously with the host cell. (4) The endosymbionts distribute to daughter cells when the host cell divides. (5) The endosymbiont has to contribute to the host's survival. Various phenomena observed in the algal reinfection process provide an excellent opportunity to elucidate these requirements.

Mixotrophy and endosymbiosis are widespread phenomena among protists than previously believed (Esteban et al., 2010). Besides *P. bursaria*, freshwater protozoa *Climacostomum virens* (Reisser, 1984, 1986), *Euplotes daidaleos* (Heckmann et al., 1983), *Vorticella* spp. (Graham and Graham, 1978, 1980), *Stentor polymorphus* (Reisser, 1984, 1986), and *Mayorella viridis* (Cann, 1981) harbour *Chlorella* spp. as their endosymbionts. Research on the endosymbiosis between *P. bursaria* and *Chlorella* spp. may lead to find a universal mechanism for establishing algae-protozoa endosymbiosis.

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