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Research Paper

Quantitative analysis of trichocysts in *Paramecium bursaria* following artificial removal and infection with the symbiotic *Chlorella variabilis*

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Abstract

Ciliates, including *Paramecium bursaria*, possess cell organelles called trichocysts that have defensive functions. *Paramecium bursaria* is capable of symbiosis with *Chlorella* sp., and the symbiotic algae are in close proximity to the trichocysts. To clarify the relationship between trichocysts in *P. bursaria* and the presence or absence of the intracellular symbiotic *Chlorella* sp., this study compared the regeneration capacity of trichocysts in alga-free and algae-bearing *P. bursaria*. In addition, trichocyst protein abundance was measured when alga-free *P. bursaria* specimens were artificially infected with *Chlorella* sp. After completely removing trichocysts from *P. bursaria* cells by treatment with lysozyme and observing them after 24 h, the percentage of regenerating trichocysts in the entire cell was significantly higher in alga-free cells than that in algae-bearing cells. There was a significant difference in the trichocyst protein abundance of *P. bursaria* before and one week after mixing with *Chlorella* sp. (i.e., after the establishment of symbiosis with the algae). We also developed a simple method for the isolation of high-purity trichocysts to quantify trichocyst protein amounts. This study shows the importance of trichocysts in alga-free *P. bursaria* as well as their competition with symbiotic *Chlorella* sp. for attachment sites during the algal infection process.

Keywords *Paramecium bursaria* · *Chlorella* sp. · endosymbiosis · quantitative analysis · trichocyst · trichocyst isolation

1. Introduction

Paramecium bursaria is a protist belonging to the highly diverse phylum Ciliophora. This broadly distributed species lives in symbiosis with green algae of the genus *Chlorella*. Interestingly, only two (*P. bursaria* and *P. chlorelligerum*) out of the twenty morphological species described in the genus *Paramecium* (Melekhin et al., 2022) are capable of symbiosis with algae (Lanzoni et al., 2016). Algae-bearing *P. bursaria* and *Chlorella* spp. have a mutually symbiotic relationship. The host *P. bursaria* supplies *Chlorella* spp. with carbon dioxide and nitrogen sources (Albers et al., 1982; Albers and Wiessner, 1985; Reisser, 1976, 1980), whereas symbiotic algae supply the host *P. bursaria* with the photosynthetic products maltose and oxygen (Brown and Nielsen, 1974; Muscatine et al., 1967; Pado, 1965). Regardless of the symbiotic relationship, algae-bearing *P. bursaria* and *Chlorella* grow independently. When algae-bearing *P. bursaria* cells were cultured under constant dark conditions or treated with the protein synthesis inhibitor cycloheximide, the symbiotic *Chlorella* sp. cells were completely removed (Kodama and Fujishima, 2008). In addition, *Chlorella*-removed cells can be re-infected with symbiotic *Chlorella* sp. isolated from algae-bearing *P. bursaria* or free-living *Chlorella* spp. (Kodama and Endoh, 2024; Kodama and Fujishima, 2009). Algae-removed *P. bursaria* cells are colorless and transparent, making it easy to observe the contents of the digestive vacuoles (DVs). Based on the above, algae-bearing *P. bursaria* and *Chlorella* spp. are considered the best model organisms for elucidating the mechanism of the establishment of intracellular symbiosis (Jenkins, 2024). Transcriptome analysis of host *P. bursaria* cells, with or without the symbiotic *Chlorella variabilis*, has also revealed changes in gene expression due to algal endosymbiosis (Kodama et al., 2014). However, the molecular mechanisms underlying the establishment and maintenance of intracellular symbiosis are largely unknown, except for those mentioned above.

Many protist species, including ciliates, have cell organelles called extrusomes (Grell, 1973; Hausmann, 1978; Rosati and Modeo, 2003). Extrusomes are arranged in the cortex immediately below the plasma membrane and are characterized by their release from the cell in response to mechanical, chemical, or electrical stimuli. By releasing these extrusomes, protists can attack or defend themselves against predators. For example, the carnivorous ciliate genera *Dileptus* (Vďačný et al., 2012) and *Didinium* (Foissner et al., 1995) have extrusomes that are known as toxicysts. Toxicysts contain toxic substances that allow them to paralyze and capture prey. In contrast, *Paramecium* and *Tetrahymena* have defensive extrusomes, called trichocysts. Trichocysts are 3–4 µm in length when intracellular, and are present in the entire cell cortex without gaps, except in the cytopharynx. However, they instantly become 25–33 µm in spear shape when released outside the cell (Adoutte, 1988; Plattner et al., 1991). Trichocysts are resynthesized several hours after their release (Harumoto, 2002). Trichocysts are associated with adhesion (Saunders, 1925), osmoregulation (Wohlfarth-Bottermann, 1953), conjugation (Vivier and Andrè, 1961), strengthening of the cell cortex (Ehret and McArdle, 1974), roles in the transport and positioning of organelles, such as new trichocysts from the cytoplasm to their ultimate locations or mitochondria within the cell (Aufderheide, 1977), and protein and amino acid storage (Haacke-Bell et al., 1990). However, the only experimentally proven function of trichocysts is a defense against certain predators (Harumoto and Miyake, 1991). Although trichocysts are generally considered to be defensive, their roles are not universally effective against all predators. For instance, they have been shown to be of little use against the predatory ciliate *Didinium nasutum*, suggesting that the defensive functions of trichocysts may be more complex and context-dependent (Miyake and Harumoto, 1996). Additionally, the evolutionary origins and biochemical makeup of trichocysts in *Paramecium* appear to be distinct from those in other lineages, such as cryptomonads, indicating a diverse evolutionary history of these organelles (Yamagishi et al., 2012).

The symbiotic *Chlorella* cells of *P. bursaria* are situated close to trichocysts. During the process of reinfection of *Chlorella* sp. with alga-free *P. bursaria*, algae are taken up through the cytopharynx and encased in the host DV membrane. Acidosomes and lysosomes fuse with the DV membrane, and *Chlorella* sp. within the DV is digested; however, some *Chlorella* cells escape digestion because they are resistant to host lysosomal digestive enzymes. Dynamin causes *Chlorella* sp. to bud out from the DV membrane and is released singly into the host cytoplasm. After release, the DV membrane enclosing a single green *Chlorella* sp. differentiates into a perialgal vacuole (PV) membrane that prevents host lysosomal fusion. Endosymbiosis is established when the PV membrane surrounding the symbiotic *Chlorella* sp. adheres to the host cell cortex (Kodama and Fujishima, 2010). As both the symbiotic *Chlorella* sp. and the host trichocysts are located just below the cell cortex of *P. bursaria*, they also compete for attachment sites (Kodama and Fujishima, 2011). In alga-free *P. bursaria* cells, many trichocysts are arranged without gaps in the cell cortex, but during the process of re-symbiosis with *Chlorella* sp., some trichocysts are digested, and *Chlorella* sp. can adhere to the gaps (Kodama and Fujishima, 2009). It has also been shown that when trichocysts are artificially removed by treating alga-free *P. bursaria* with lysozyme and then adding *P. bursaria* with *Chlorella* sp., the algae can adhere more densely to the *P. bursaria* cell cortex (Kodama and Fujishima, 2009). Transmission electron microscopy has also revealed that trichocyst density is higher in alga-free *P. bursaria* than in algae-bearing ones (Kodama and Fujishima, 2022; Omura and Suzaki, 2003). Furthermore, it has been found that algae-bearing *P. bursaria* digest intracellular *Chlorella* sp. and resynthesize trichocysts when they are starved (Kodama and Fujishima, 2009; Kodama and Miyazaki, 2021). Thus, several previous studies have clearly shown that symbiotic *Chlorella* spp. affect the number and arrangement of host trichocysts during the process of re-symbiosis and maintenance of symbiotic algae.

To elucidate the function of trichocysts in *P. bursaria*, we artificially removed trichocysts from algae-bearing and alga-free *P. bursaria* and compared their regeneration rates. A method for isolating trichocysts with high purity was established to quantify trichocyst protein amounts, and their purity was examined by indirect immunofluorescence microscopy using a monoclonal antibody that specifically recognizes *P. bursaria* trichocysts. Furthermore, the protein abundance of trichocysts during the algal infection process was quantified.

2. Material and methods

2.1. Cultivation of alga-free and algae-bearing P. bursaria

Two *P. bursaria* strains were used in this study: alga-free Yad1w and algae-bearing Yad1g1N (syngen R3, mating type III). The Yad1g1N strain was produced by infecting cloned symbiotic *Chlorella variabilis* strain 1 N cells with Yad1w cells (Kodama and Fujishima, 2011). *Paramecium* cell culture, prepared using red pea (*Pisum sativum*) extract culture medium (Tsukii et al., 1995) in modified Dryl's solution (MDS) ($NaH_2PO_4 \cdot 2H_2O$ replaced with KH_2PO_4) (Dryl, 1959), was inoculated with non-pathogenic *Klebsiella aerogenes* (ATCC35028) as food bacteria 1 d before use

(Fujishima et al., 1990). For ordinary cultures, several hundred *P. bursaria* cells were used to inoculate 2 ml aliquots of the culture medium in test tubes. Subsequently, 2 ml of fresh culture medium was added daily for 12 d. Cultures in the early stationary phase were used in experiments 1 d after the last feeding and were cultured at 23 ± 1 °C. Algae-bearing *P. bursaria* cells were cultured under fluorescent light maintained at 20–30 μmol photons $m^{-2}s^{-1}$ using fluorescent light (27 W, natural white) under constant light (24L:0D).

2.2. Preparation of trichocysts-free P. bursaria cells

Lysozyme can induce massive discharge of trichocysts in *P. bursaria* without seriously injuring the cells (Kodama and Fujishima, 2009). The number of algae-bearing and alga-free *P. bursaria* cells was adjusted to 5000 cells/ml after washing with MDS. Stock solutions of lysozyme (10 mg/ml in 10 mM Na-PB, pH 6.8) (Sigma-Aldrich Corp., Japan) were stored at −20 °C until use. The stock solution was diluted with MDS to obtain a final lysozyme concentration of 1 mg/ml, and algae-bearing and alga-free *P. bursaria* cells were treated with lysozyme at 23 ± 1 °C under fluorescent light for 5 min. The discharged trichocysts were then washed using a centrifuge tube equipped with a 15 μm pore nylon mesh by pouring 100 ml of MDS. Immediately and 24 h after lysozyme treatment, cell suspensions were mixed with an equal volume of saturated picric acid solution. Picric acid is a strong inducer of trichocyst discharge in *Paramecium*, simultaneously fixing cells at the same time (Pollack, 1974). The trichocyst-discharged or regenerated areas were then categorized by manual microscopic identification. The cells were divided into four areas as shown in Figure 1A. We categorized A if trichocyst discharge or regeneration from the anterior cortex was observed, VD if from the ventral or dorsal cortex, P if from the posterior cortex, O if from the whole cell, and N if no discharge or regeneration was observed.

2.3. Isolation of symbiotic C. variabilis from algae-bearing P. bursaria

To isolate the symbiotic *C. variabilis* strain 1N cells, 300 ml of cell cultures of algae-bearing *P. bursaria* Yad1g1N were strained through two layers of KimWipes to remove gross debris and subsequently filtered using a centrifuge tube equipped with a 15 μm pore nylon mesh. Yad1g1N cells were washed with 50 ml of MDS, harvested using a hand-operated centrifuge (UKG-2; Uchida Rikakiki, Tokyo, Japan), and resuspended in 1 ml of MDS. The sedimented cells were suspended in 0.1 mM phenylmethylsulphonyl fluoride (Sigma-Aldrich, St. Louis, MO, USA) containing 1 ml MDS and manually homogenized in a Teflon homogenizer on ice with 30 strokes. The homogenates were then filtered using a centrifuge tube equipped with a 15 μm nylon mesh. The filtrate was transferred to 1.5 ml tubes, washed three times with 1.5 ml MDS via centrifugation at 4350 *g* for 1 min, and then reduced to a final volume of 500 μl. Algal cell density was calculated using a Thoma blood-counting chamber.

2.4. Infection of alga-free P. bursaria cells with symbiotic C. variabilis

The isolated symbiotic *C. variabilis* 1N was mixed with alga-free *P. bursaria* (5×10^7 algal cells mixed with 5×10^3 paramecia/ml) in a 50 ml conical flask at 20–30 μmol photons $m^{-2}s^{-1}$ using fluorescent light (27 W, natural white) under constant light (24L:0D). 24 h after mixing, the paramecia were separated from the uningested algae using a 15 μm pore size nylon mesh by pouring MDS. The paramecia retained in the mesh were transferred to a conical flask and resuspended in MDS. Before, 24 h, 48 h, and 1 week after algal mixing, a 100 µl aliquot of the cell suspension was fixed with 100 µl of 8 % (w/v) paraformaldehyde (PFA). After 48 h of mixing with algae and alga-free *P. bursaria*, food bacteria were added to the culture every alternate day. The fixed cells were observed using a differential interference contrast (DIC) microscope (BX53, EVIDENT, Tokyo, Japan). Images were captured using a DP74 microscope (EVIDENT, Tokyo, Japan).

2.5. Isolation of trichocysts from alga-free P. bursaria by stepwise density-gradient centrifugation using sucrose

Approximately 600 ml of culture medium of alga-free *P. bursaria* strain Yad1w in the stationary phase of growth was strained through two layers of KimWipes to remove gross debris and subsequently filtered using a centrifuge tube equipped with a 15 µm pore nylon mesh. Yad1w cells were washed with 50 ml of MDS, harvested using a hand-operated centrifuge, and resuspended in 1 ml of MDS (Fig. 2A). The 1 ml of cell suspension was washed once with 10 ml of TSCM buffer (10 mM Tris-HCl, pH 7.9, 0.25 M sucrose, 3 mM $CaCl_2$, 8 mM $MgCl_2$, Dohra et al., 1994). 1 ml of TSCM buffer containing 0.1 mM PMSF was added to the cell suspension and homogenized in a Teflon homogenizer on ice. The cells were disrupted completely, and the homogenates were centrifuged by stepwise density-gradient centrifugation using 2 ml of each concentration of sucrose (2.0, 1.6, 1.3, and 1.0 M) at 700 *g* for 10 min at 4 °C in a 10 ml centrifuge tube. The boundary surface of each concentration was carefully aspirated using a Pasteur pipette and observed under a DIC microscope. The experimental procedure is illustrated in Figure 2B.

2.6. Simplified isolation method of trichocysts from alga-free P. bursaria by using saturated picric acid

One milliliter of the cell suspension of Yad1w was obtained as described above, with the exception that MDS was used instead of TSCM (Fig. 2A). From the cell suspension, 50,000 cells were collected and added to a 1.5 ml micro-centrifuge tube. Equal volumes of saturated picric acid were mixed with the cell suspension to discharge trichocysts. Picric acid is known to induce the complete release of trichocysts (Galvani and Sperling, 2000). Treatment with saturated picric acid induced the immediate release of trichocysts from Yad1w cells and fixation of the cells. The mixture of *P. bursaria* and picric acid was gently pipetted with a 200 µl micropipette to avoid cell disruption. The tube was centrifuged using a hand-operated centrifuge, and the supernatant, which contained many discharged trichocysts, was transferred to a new 1.5 ml tube. To harvest any remaining trichocysts, 100 µl of MDS was added to the remaining precipitate, centrifuged again using a hand-operated centrifuge, and the supernatant was collected in the above-mentioned 1.5 ml tube. The tube was then centrifuged at 20,000 *g* for 10 min at 4 °C. Trichosyst sedimentation was repeatedly washed with 100 µl of MDS until the yellow color of picric acid was no longer visible under the same centrifugation conditions. The experimental procedure is illustrated in Figure 2C.

2.7. Indirect immunofluorescence microscopy

Aliquots of trichocysts isolated by the simplified isolation method, shown in Figure 2C, were air-dried on cover glasses (4.5 × 24.0 mm). The trichocysts were then fixed with 4 % (w/v) paraformaldehyde in phosphate-buffered saline (PBS) (137 mM NaCl, 2.68 mM KCl, 8.1 mM $NaHPO_4 \cdot 12\ H_2O$, 1.47 mM KH_2PO_4, pH 7.2) for 10 min at 4 °C. The fixed trichocysts were washed with PBS containing 0.05 % Tween 20 (PBST) and PBS for 10 min at 4 °C. The trichocysts were then treated with monoclonal antibodies (mAbs) against *Paramecium* trichocysts (Kodama and Fujishima, 2009) overnight at 4 °C. The trichocysts were washed twice with PBS. Next, the trichocysts were treated with Alexa Fluor 488 goat anti-mouse IgG (Molecular Probes) diluted 1000-fold in PBS for 2 h at 23 ± 1 °C and washed twice with PBS for 10 min. The samples were observed under a DIC and fluorescence microscope (BX53, EVIDENT, Tokyo, Japan) equipped with fluorescence mirror units of U-FBNA (excitation 470–495 nm, emission 510–550 nm) for Alexa Fluor 488. Images were acquired as previously described.

2.8. Isolation of trichocysts from P. bursaria cells

To examine trichocysts of *P. bursaria* cells during the algal reinfection process, trichocysts were isolated from 50,000 *P. bursaria* cells before and 24 h, 48 h, and 1 week after algal mixing at each time. Trichocysts were isolated by using the simplified isolation method shown in Figure 2C. We confirmed that picric acid treatment resulted in the complete discharge of trichocysts from both alga-free and algae-bearing *P. bursaria* (Suppl. Fig. S1).

2.9. Extraction of trichocyst protein

To obtain trichocyst protein extracts, trichocysts isolated from *P. bursaria* cells during the algal reinfection process were lysed using the EzRIPA Lysis kit (ATTO, Tokyo, Japan) according to the manufacturer’s protocol. After the addition of 50 µl of RIPA Lysis buffer to the trichocyst sedimentation, they were incubated on ice for 15 min. The lysate was observed under a DIC microscope to confirm the complete dissolution of the trichocysts. The solution was then centrifuged at 14,000 *g* for 10 min. The centrifuged supernatant was transferred to a new microcentrifuge tube and stored at −80 °C until use.

2.10. Quantification of protein abundance in trichocysts isolated from P. bursaria

The protein abundance in trichocysts isolated from *P. bursaria* cells before and 24 h, 48 h, and 1 week after algal mixing was quantificated using a TaKaRa BCA Protein Assay Kit (Takara Bio, Shiga, Japan). Calibration curves were generated using a PiCOEXPLORER (PAS-110-YU, Yamato Scientific Co., Ltd., Japan) with a color sensor R (wavelength range: 575–660 nm).

2.11. Quantification and statistical analysis

Welch's *t*-test and two-sided Fisher's exact test were used for the statistical evaluation of the results. Values for $p < 0.05$ were considered statistically significant. All data are presented as mean ± standard deviation (SD). All statistical analyses were performed using the R software (R ver 4.1.3) following Ihaka and Gentleman (1996).

3. Results

3.1. Comparison of trichocyst regeneration of alga-free and algae-bearing P. bursaria cells

The degree of trichocyst removal and regeneration in *P. bursaria* cells with or without symbiotic algae, immediately and 24 h after trichocyst removal, was divided into five categories, as described by Kodama (2013) (Fig. 1A). As shown in the left graph in Figure 1B, all trichocysts could be removed by treatment with 1 mg/ml lysozyme for 5 min in both alga-free and algae-bearing *P. bursaria*, as evidenced in that none of the observed cells discharged trichocysts (N). The right graph in Figure 1B shows the percentage of trichocyst regeneration from the five categories as shown in the legend. In the case of algae-bearing *P. bursaria*, 6 % of observed cells regenerated trichocysts in the anterior cortex (A), 15 % of observed cells regenerated trichocysts in the ventral or dorsal cortex (VD), 12 % of observed cells regenerated trichocysts in the posterior cortex (P), 31 % of observed cells regenerated trichocysts overall (O), and 36 % of observed cells did not regenerate trichocysts (N). The percentages of alga-free *P. bursaria* were 13, 5, 6, 56, and 20 %, respectively. The percentages of O in algae-bearing and alga-free *P. bursaria* were 31 and 56 %, respectively. A Fisher's exact probability test using these data showed a significant difference ($p < 0.01$). This experiment showed that alga-free *P. bursaria* cells regenerated trichocysts more rapidly than algae-bearing *P. bursaria* cells 24 h after trichocysts removal.

3.2. Isolation of trichocysts of alga-free P. bursaria by stepwise density-gradient centrifugation using sucrose

To quantify the amount of trichocyst proteins, it is necessary to develop a method for isolating high-purity trichocysts. First, trichocyst isolation was performed by stepwise density-gradient centrifugation with sucrose. Numerous microscopic organelles with diameters of a few micrometers were observed at the interface between the *P. bursaria* homogenate and 1.0 M sucrose, but no trichocysts were identified (Fig. 3A). A small number of trichocysts were observed on the boundary surface of 1.0 and 1.3 M (Fig. 3B). In addition, numerous aggregates of cell membrane fragments and cilia were observed. At the boundary surface of 1.3 and 1.6 M, trichocysts, intracellular crystals, and small vesicles such as acidosomes and lysosomes were observed (Fig. 3C). Several cell membrane fragments were found at this boundary as well. At the boundary surface of 1.6 and 2.0 M, many crystals and macronucleus were observed, but no trichocysts were detected (Fig. 3D). Thus, trichocysts could be identified at sucrose concentrations of 1.0, 1.3, and 1.6 M, but many other organelles were also mixed.

3.3. Indirect immunofluorescence microscopy of trichocysts isolated by simplified method from alga-free P. bursaria

Trichocysts isolated using the method shown in Figure 2C were subjected to indirect immunofluorescence microscopy using an anti-*Paramecium* trichocyst monoclonal antibody and observed using DIC and fluorescence microscopy. High-density trichocysts were isolated with high purity and no cell membrane fragments or crystals were observed (Fig. 4A). Fluorescence microscopy images also revealed the isolation of high-density and high-purity trichocysts (Fig. 4B). Trichocyst matrix protein called "body" at the tip of some trichocysts was identified by immunofluorescence (Fig. 4B, arrowhead). This new trichocyst isolation method was more efficient, faster, and more purified than conventional methods such as stepwise density-gradient centrifugation using sucrose. The advantages of this procedure are that it is easier than conventional isolation methods and does not involve cell disruption; therefore, contamination of other organelles is very small. In addition, since almost no trichocysts were found in the pellets of alga-free *P. bursaria* after trichocyst isolation, the loss of trichocysts by this procedure was quite small.

3.4. Quantification of trichocyst protein abundance during the algal infection process

We measured how trichocyst protein abundance changed during the algal infection process in alga-free *P. bursaria* cells. The trichocyst protein abundance of alga-free *P. bursaria* before mixing with *Chlorella* sp. (Fig. 5A, upper left panel), 24 h after mixing with algae (Fig. 5A, upper right panel), 48 h after mixing with algae (Fig. 5A, lower left panel), and 1 week after mixing with algae (Fig. 5A, lower right panel) were 981.7 mg/ml, 796.8 mg/ml, 577 mg/ml, and 254.8 mg/ml, respectively (Fig. 5B). The adhesion of a single green *Chlorella* cell beneath the *P. bursaria* cell cortex was observed 24 h after mixing (Fig. 5A, upper right panel, arrowheads). This indicated that re-symbiosis of *Chlorella* sp. with the host *P. bursaria* was established. One week later, *Chlorella* sp. was present throughout the host cytoplasm and adhered to the host cell cortex layer without gaps, indicating that symbiosis was maintained, and the abundance of *Chlorella* specimens increased by cell division within the host cells (Fig. 5A, lower right panel). The Mann-Whitney two-tailed U test showed significant differences ($p < 0.05$) in the amount of protein before and after 1 week of algal mixing (Fig. 5B, asterisk). Thus, the establishment of algal endosymbiosis in alga-free *P. bursaria* reduced the trichocyst protein abundance.

4. Discussion

The importance of studying the effects of symbionts on the number and role of organelles in host protists lies in understanding the intricate relationships that define symbiotic interactions and their effects on host cellular physiology. As symbionts alter gene expression, contribute to metabolic processes, and influence host immune responses (Wang et al., 2023), they may affect host organelle function and number via various mechanisms. For instance, the presence of symbiotic bacteria in the social amoeba *Dictyostelium discoideum* has been associated with genomic adaptations that may influence host–symbiont interactions, including the potential modification of organelle function (Noh et al., 2022).

The isolation of trichocysts has been reported in several studies (Glas-Albrecht et al., 1992; Lima et al., 1989). These methods require the separation of trichocysts from other organelles for cell destruction by homogenization. Furthermore, percoll, an expensive reagent, was used for centrifugation. However, the method shown in Figure 2C and developed in this study does not crush cells; therefore, there is no contamination by organelles other than trichocysts. In addition, inexpensive picric acid is used to release trichocysts. Therefore, this method is useful for isolating trichocysts only.

As shown in Figure 1B, 24 h after artificial trichocyst removal, alga-free *P. bursaria* cells regenerated trichocysts more rapidly and in larger quantities than did algae-bearing cells. There are three possible reasons for this finding. First, alga-free *P. bursaria* cells may regenerate trichocysts at a faster rate to maintain cell strength because one of the functions of trichocysts is to maintain the strengthening of the cell cortex (Ehret and McArdle, 1974). In algae-bearing *P. bursaria* cells, 36 % of cells did not regenerate trichocysts. Symbiotic algae have a rigid cell wall that may strengthen the surface structure of *P. bursaria* instead of trichocysts. Second, algae-bearing *P. bursaria* cells have a large amount of symbiotic *Chlorella* sp. adhering to the cell membrane cortex; therefore, there may be little space for trichocysts to adhere to. Kodama and Fujishima (2009) found that artificially removing trichocysts increases the space for symbiotic *Chlorella* sp. to attach to the cell cortex and increases the number of symbiotic algae in the host. Kodama and Fujishima (2009) also showed that the number of trichocysts in the cell cortex increased as the number of symbiotic *Chlorella* sp. decreased when *Chlorella* sp. cells were removed from algae-bearing *P. bursaria* using cycloheximide, which preferentially inhibits protein synthesis in *Chlorella* sp. Thus, the resynthesis of trichocysts in algae-bearing *P. bursaria* cells is slower than in alga-free cells because there is less space for trichocysts to reattach. Third, Berger (1980) suggested that *Didinium nasutum*, a predator of *Paramecium* spp., showed a preference for attacking and ingesting alga-free *P. bursaria* compared to algae-bearing ones. The symbiotic algae within *P. bursaria* are hypothesized to release distasteful metabolites that repel *D. nasutum*, thereby providing a protective function to the host (Berger, 1980). As the primary function of trichocysts is protection from predators, alga-free *P. bursaria* may need to regenerate trichocysts as soon as possible to escape predation. However, further experiments are required to confirm this hypothesis.

While previous studies have shown that the number of host trichocysts decreases with *Chlorella* sp. symbiosis (Kodama and Fujishima, 2011) and that the amount of host mitochondrial protein also decreases after *Chlorella* sp. symbiosis (Kodama and Fujishima, 2022), this study is the first to reveal the timing of the decrease in trichocyst protein abundance during algal infection process (Fig. 5B). Results in this study support with quantitative data the findings of Kodama and Fujishima (2011, 2023) at the microscopic level that symbiotic *Chlorella* sp. adhere to the attachment sites of existing trichocysts and reduce trichocysts. As mentioned in the ‘Introduction’ section, *P. bursaria* and symbiotic *Chlorella* spp. have long been known to have a mutual relationship, and many previous studies have focused on the benefits of symbiotic algae to host cells. It has been found that algae-bearing *P. bursaria* are more tolerant to various stresses, such as high temperature, oxidation, and UV radiation, when they retain symbiotic *Chlorella* spp. (Kobayashi and Miwa, 2011). In addition, transcriptome analysis of the host *P. bursaria* with or without symbiotic *Chlorella* sp. has shown that HSP70 is highly expressed in host cells with symbiotic algae compared to those without algae (Kodama et al., 2014). In contrast, algal endosymbiosis decreases the number of intracellular trichocysts. Since trichocysts are cellular organelles with defensive functions, we hypothesized that while the benefits of symbiotic *Chlorella* spp. would be great in a laboratory environment with good culture conditions and no external enemies, the disadvantages of losing trichocysts would be greater in outdoor environments. In fact, some *P. bursaria* collected from outside had only a few symbiotic algae (Morita and Kodama, Fujishima and Kodama, unpublished observations). It is also known that *P. bursaria* intermittently retains intracellular symbiotic algae and the cell division cycles of the host and symbiotic *Chlorella* spp. are synchronized (Kadono et al., 2004; Takahashi et al., 2007). *Paramecium bursaria* specimens with few *Chlorella* cells are very likely able to control the division of symbiotic algae, which, in turn, prevents the reduction of the trichocyst number. The effects of reduced numbers of trichocysts on the host *P. bursaria* have not been investigated hitherto. When algae-bearing *P. bursaria* are cultured under constant dark and starvation conditions, they digest symbiotic algae and use their nutrients for trichocyst resynthesis (Kodama and Miyazaki, 2021). Thus, the resynthesis of trichocysts seems to be a priority even under starvation conditions, suggesting that these extrusive organelles are of high importance for *P. bursaria*.

5. Conclusion

The results of this study can be summarized as follows. Alga-free *P. bursaria* cells resynthesized trichocysts more rapidly than algae-bearing cells when artificially removed. Conversely, trichocysts were reduced during the algal infection process within 1 week after algal mixing. These findings suggest a multifaceted role of trichocysts in the environment and their importance for the ciliate host. Studies on trichocysts can help to reveal the defense strategies of ciliates, mechanisms of predator–prey interactions, and complex relationships between cellular organelles and symbiotic partners. We hope that the method for isolation of trichocysts, developed in the present study, will contribute to the elucidation of trichocyst functions.

CRediT authorship contribution statement

Hikaru Morita: Data curation, Formal analysis, Investigation, Visualization, Writing – original draft. **Yuuki Kodama:** Data curation, Formal analysis, Investigation, Visualization, Supervision, Funding acquisition, Project administration, Writing – review & editing.

Data availability

The datasets used in this study are available from the corresponding author upon reasonable request.

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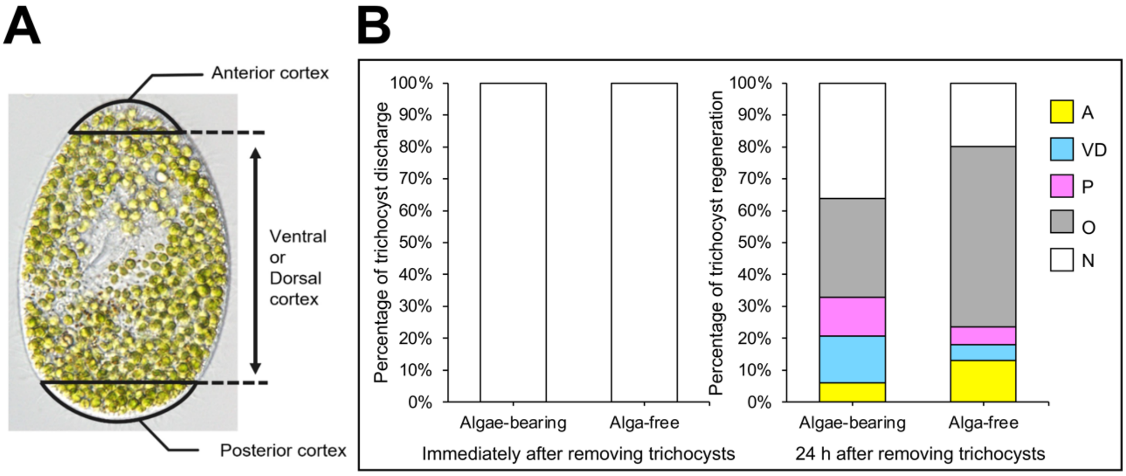
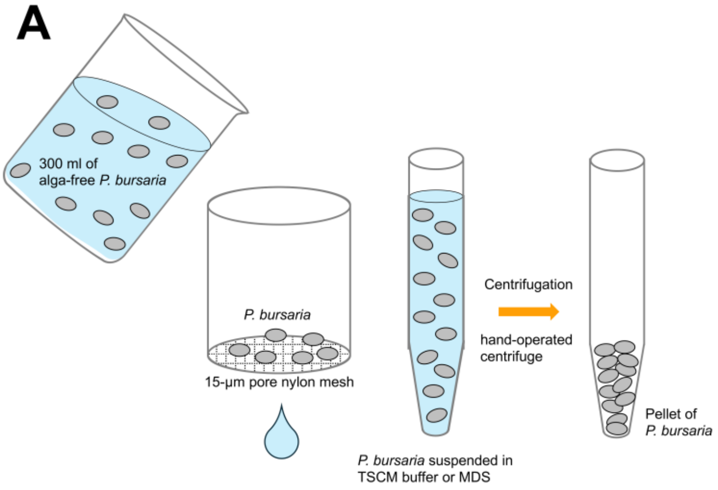
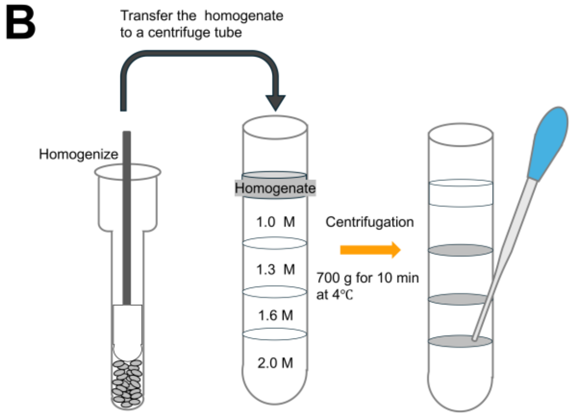


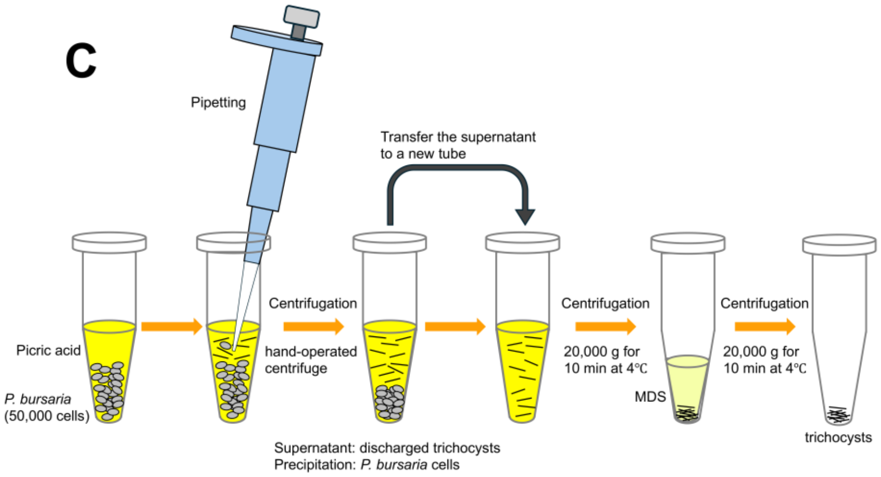
Fig. 1. Comparison of the amount and rate of trichocyst discharge and regeneration in alga-free and algae-bearing *P. bursaria*. (**A**) Photomicrograph of an algae-bearing *P. bursaria* specimen, strain Yad1g1N. For the purpose of this study, the cortex was divided into three parts: the anterior cortex, the posterior cortex, and the ventral or dorsal cortex. (**B**) Percentage of trichocyst discharge or regeneration: A indicates trichocyst regeneration at the anterior cortex, VD either at the ventral or the dorsal cortex, P at the posterior cortex, O underneath the whole cell surface, and N indicates that no regeneration was observed. Algae-bearing and alga-free on the *x*-axis mean data for algae-bearing and alga-free *P. bursaria*, respectively. The graph on the left documents that all trichocysts of algae-bearing and alga-free *P. bursaria* cells were successfully removed. The graph on the right shows the percentage of trichocysts regenerated 24 h after their removal. The highest percentage of O (regenerated underneath the whole cell surface) was observed in alga-free *P. bursaria*, while the highest percentage of N (no regeneration) was observed in algae-bearing *P. bursaria*. Six replicates were analyzed and 271–787 cells were observed.



Concentration of alga-free *P. bursaria* cells



Isolation of trichocysts by sucrose density gradient centrifugation



Isolation of trichocysts using saturated picric acid

Fig. 2. Schematic representation of the method for isolating trichocysts from *P. bursaria.* After enrichment of alga-free *P. bursaria* (**A**), trichocysts were isolated by the conventional method using sucrose density gradient centrifugation (**B**). In the present study, trichocysts were released from alga-free *P. bursaria* via stimulation with saturated picric acid (**C**). For further details, see the 'Material and methods' section.

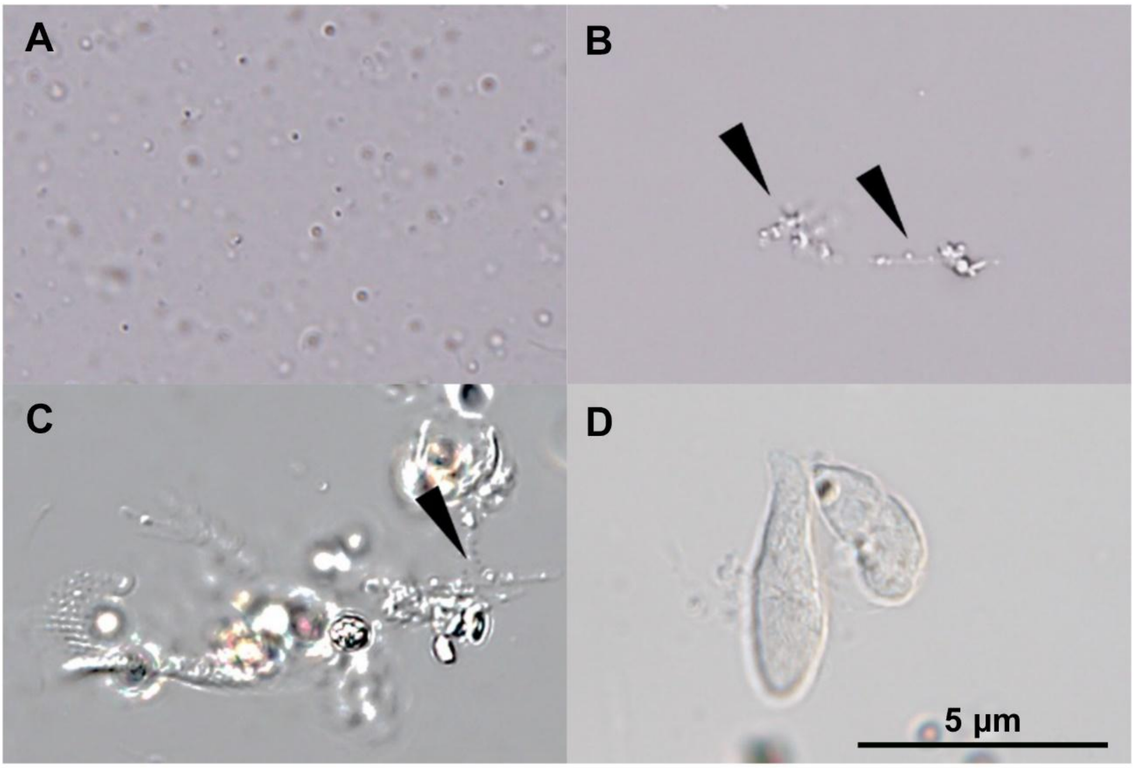


Fig. 3. Isolation of trichocysts by sucrose density gradient centrifugation. (**A**) Contents of the boundary surface of the cell homogenate and 1.0 M sucrose. Microscopic organelles of several micrometers in size were visible; however, trichocysts were not observed. (**B**) To show the contents of the boundary surface of 1.0 and 1.3 M sucrose. Trichocysts (arrowheads) were observed. Cell membrane debris and cilia were detected as well. (**C**) To show the content of the boundary surface of 1.3 and 1.6 M sucrose. More trichocysts were observed here than in (B). Cell membrane fragments and crystals

were also found. (**D**) To show the content of the boundary surface of 1.6 and 2.0 M sucrose. A large number of crystals and macronucleus were observed, but no trichocysts were detected.

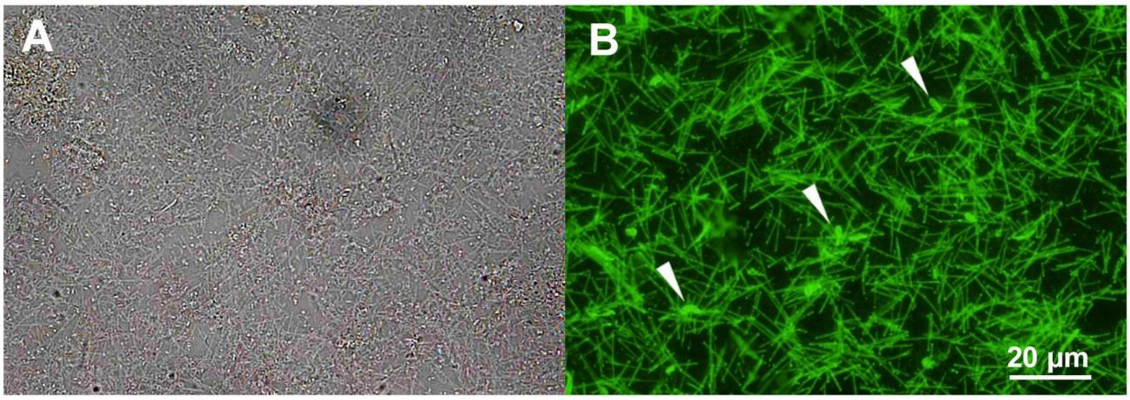


Fig. 4. Photomicrographs of indirect immunofluorescence microscopy of trichocysts isolated using saturated picric acid. (**A**) Trichocysts isolated using the simplified isolation method, as shown in Figure 2C, were observed using DIC microscopy. Because the trichocysts are colorless and transparent, the contrast of the photomicrograph was increased so that they could be seen. Numerous linear trichocysts are recognizable. Compared to the method shown in Figure 2B, there is much less contamination by other organelles. (**B**) Indirect immunofluorescence micrograph of (A). The immunofluorescence of goat anti-mouse IgG was observed. Some trichocyst matrix proteins (arrowheads) are visible.

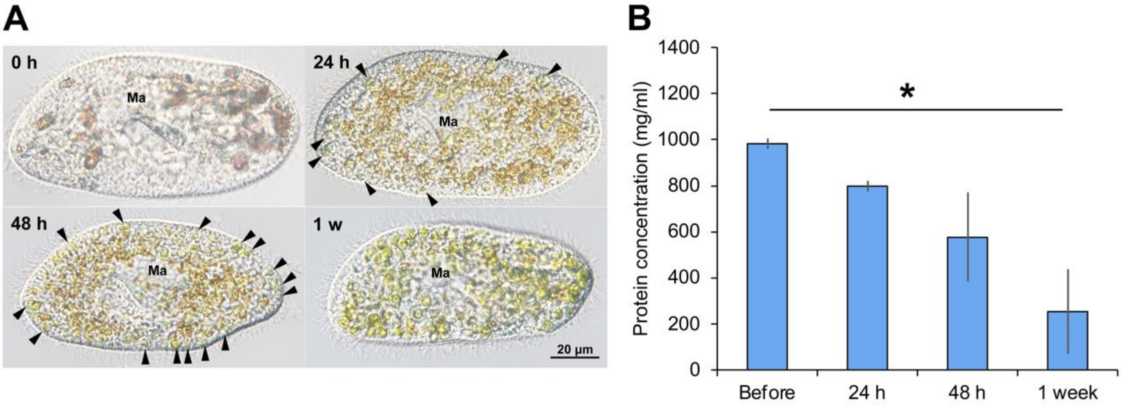


Fig. 5. Trichocyst protein abundance during infection of alga-free *P. bursaria* with *Chlorella* sp. (**A**) Photomicrographs of alga-free *P. bursaria* during algal reinfection 0 h, 24 h, 48 h, and 1 week after algal mixing. Before (0 h) mixing with algae, many crystals (reddish-brown structures) were observed in the cells. 24 h after mixing, many algae were ingested by *Paramecium*. Although numerous digested brown algae were noted, some green algae localized beneath the host cell cortex were also observed (arrowheads), which established endosymbiosis. 48 h after mixing, green algae, which were localized beneath the host cell cortex (arrowheads), were observed. Digested brown algae were still recognizable. One week after mixing, the number of green symbiotic algae increased by cell division. In addition, the green color of the algal chloroplasts became darker. No brown-digested *Chlorella* sp. was observed. Ma, macronucleus. (**B**) Changes in trichocyst protein abundance during *Chlorella* sp. reinfection. The *y*-axis indicates protein abundance and the *x*-axis indicates elapsed time. Significant differences were observed in trichocyst protein abundance before (0 h) and 1 week after algal mixing. Error bars indicate standard deviations (SD) and asterisks indicate significant differences (Mann–Whitney U test, * $p < 0.5$).

Fig. S1. Photomicrographs of *P. bursaria* with and without algae after treatment with picric acid. Algae-bearing *P. bursaria* (g) and alga-free *P. bursaria* (w) were washed with Dryl's solution to obtain a cell suspension. Equal amounts of picric acid were mixed and immediately observed under a DIC microscope. As a result, a large amount of spear-shaped trichocyst release was observed from the whole cells with and without algae (**A**). Picric acid treatment caused a yellowish coloration of the cells, which made it difficult to observe symbiotic algae in algae-bearing cells. Therefore, photomicrographs were obtained in (**B**) by changing the focus from (A). As a result, a large number of spherical symbiotic algae was observed in (g).