



島根大学学術情報リポジトリ
S W A N
Shimane University Web Archives of kNowledge

Title

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

Author(s)

Yuuki Kodama, Masahiro Fujishima

Journal

FEMS Microbiology Letters, Volume 370, 2023, fnad088

Published

02 September 2023

URL (The Version of Record)

<https://doi.org/10.1093/femsle/fnad088>

この論文は出版社版ではありません。
引用の際には出版社版をご確認のうえご利用ください。

This version of the article has been accepted for publication,
but is not the Version of Record.



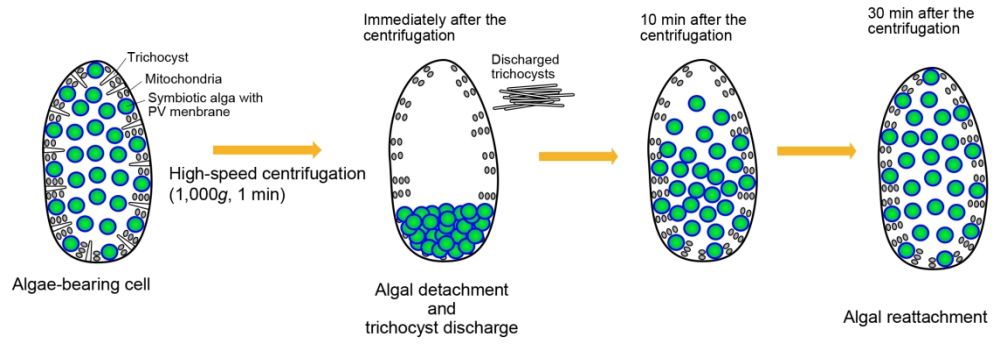
<http://mc.manuscriptcentral.com/fems>

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

Journal:	<i>FEMS Microbiology Letters</i>
Manuscript ID	FEMSLE-23-03-0064.R2
Manuscript Type:	Research Letter
Keywords:	Algal reattachment, <i>Chlorella variabilis</i> , Endosymbiosis, Mitochondria, <i>Paramecium bursaria</i> , Trichocyst
Please select the most appropriate subject section for your submission from the drop down list. For full section descriptions please refer to the Author guidelines .	Environmental Microbiology and Microbial Ecology (Editor: Tim Daniell)

SCHOLARONE™
Manuscripts

1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17
18
19
20
21
22
23
24
25
26
27
28
29
30
31
32
33
34
35
36
37
38
39
40
41
42
43
44
45
46
47
48
49
50
51
52
53
54
55
56
57
58
59
60



183x66mm (300 x 300 DPI)

1
2
3
4
5
6 1 **Role of host ciliate *Paramecium bursaria* mitochondria and trichocysts for**
7
8 2 **symbiotic *Chlorella variabilis* attachment beneath the host cell cortex**
9

10
11 3
12
13 4 Mitochondria and trichocysts of the ciliate *Paramecium bursaria*
14
15
16 5

17
18 6 **One sentence summary:** Although the host mitochondria of ciliate *Paramecium*
19
20 7 *bursaria* are near symbiotic algae during reattachment, this does not mean that the algae
21
22 8 can exist if mitochondria are present, indicating some localization bias within the host
23
24 9 cell.
25
26
27
28
29
30

31 11 **Abstract**
32

33 12 Symbiotic *Chlorella variabilis* is encased in the perialgal vacuole (PV) membrane of
34
35 13 ciliate *Paramecium bursaria*. The PV membrane is stably anchored below the host cell
36
37 14 cortex by adhesion to host mitochondria. Host trichocysts, which are defensive
38
39 15 organelles against predators, are present in the mitochondria and PV membrane vicinity.
40
41 16 The mechanism by which PV attaches beneath the host cell cortex remains unknown.
42
43 17 When *P. bursaria* is centrifuged at high speed, the symbiotic algae are displaced from
44
45 18 the host cell cortex and concentrate at the posterior end. When centrifugation is stopped,
46
47 19 the dislocated algae reattach beneath the host cell cortex with fast cytoplasmic
48
49 20 streaming. The densities of mitochondria and trichocysts before and after centrifugation
50
51 21 were compared using indirect immunofluorescence microscopy with monoclonal
52
53 22 antibodies. Almost all trichocysts were shed by high-speed centrifugation, but
54
55
56
57
58
59
60

1
2
3
4
5
6 23 dislocated algae could reattach even in the absence of trichocysts. In contrast, host
7
8 24 mitochondria were unaffected in localization and number, and the dislocated algae also
9
10 25 reattached. These findings suggest trichocysts are unnecessary for algal re-localization
11
12 26 and that mitochondria are colocalized with the algae. However, many mitochondria
13
14 27 were also present in the cell's anterior region without symbiotic algae. Therefore, not all
15
16 28 areas with mitochondria contained algae, but there was a localization bias within the
17
18 29 host cell.
19
20
21
22
23
24
25

26 31 **Keywords:** Algal reattachment, *Chlorella variabilis*, Endosymbiosis, Mitochondria,
27
28 32 *Paramecium bursaria*, Trichocyst
29
30

31 33

34 Introduction

35 35 *Paramecium bursaria*, a freshwater ciliate, is a symbiotic organism that establishes
36
37 36 endosymbiotic relationships with *Chlorella* spp. Each symbiotic alga is enclosed in a
38
39 37 perialgal vacuole (PV) derived from the host digestive vacuole (DV), which protects the
40
41 38 alga from lysosomal fusion (Gu *et al.* 2002; Karakashian and Rudzinska 1981). This
42
43 39 relationship between ciliates and algae is mutualistic; the host cell supplies algae with
44
45 40 nitrogen and oxygen (Albers *et al.* 1982, 1985; Reisser 1976, 1980), whereas the algae
46
47 41 provide the host with photosynthetic products (Brown *et al.* 1974; Reisser 1986) and
48
49 42 CO₂ (Reisser 1980). However, both *P. bursaria* and symbiotic algae can live without
50
51 43 their partners. The re-establishment of endosymbiosis between algae-free (removed) *P.*
52
53 44 *bursaria* cells and symbiotic *Chlorella* cells isolated from algae-bearing hosts can be
54
55
56
57
58
59
60

1
2
3
4
5
6 45 induced (Kodama and Fujishima 2010). In addition to their original symbiotic algae,
7
8 46 algae-free *P. bursaria* cells can be reinfected with bacteria and yeast that are retained in
9
10
11 47 the cytoplasm (Görtz 1982; Watanabe *et al.* 2022). The nuclear genomes of the
12
13 48 symbiotic *Chlorella variabilis* (Blanc *et al.* 2010) and the host *P. bursaria* have been
14
15 49 explored (Cheng *et al.* 2020); these organisms are now considered models for
16
17
18 50 endosymbiosis research.

21 Using pulse-labeling and chasing of algae-free paramecia for 1.5 min with
22
23 52 symbiotic algae that were isolated from algae-bearing *P. bursaria*, four important
24
25 53 cytological events have been identified. These events are necessary for the
26
27
28 54 establishment of endosymbiosis and the timing of each mechanism during the infection
29
30
31 55 process (Kodama and Fujishima 2010). 1) Within 3 min of algal mixing, part of the
32
33 56 algae has resistance to the host's lysosomal digestive enzymes in the DVs. 2) Within 30
34
35
36 57 min of mixing, algae in the DV begin budding from the DV membrane into the
37
38
39 58 cytoplasm. 3) Within 15 min of budding from the DV, the DV membrane enclosing a
40
41 59 single green *Chlorella* differentiates into a PV membrane. 4) The PV membrane
42
43
44 60 translocates beneath the host cell cortex.

46 61 The PV appears to localize near the host mitochondria and trichocysts. In previous
47
48 62 studies, monoclonal antibodies against *P. bursaria* trichocysts and mitochondria were
49
50
51 63 obtained; both mitochondrial and trichocyst densities of algae-bearing *P. bursaria* were
52
53 64 significantly lower than those of algae-free cells (Kodama and Fujishima 2011, 2022).
54
55
56 65 These results indicate that symbiotic algae compete for their attachment sites with
57
58
59 66 preexisting trichocysts and mitochondria, and algae have the ability to ensure algal
60

1
2
3
4
5
6 67 attachment sites beneath the host cell cortex (Kodama and Fujishima 2011).
7
8 68 Furthermore, high-speed centrifugation can induce rapid algal detachment from the host
9
10
11 69 cell cortex and concentrates the algae in the posterior end of the host cell (Kodama and
12
13
14 70 Fujishima 2013). Within 10 min of centrifugation, the detached algae recover their
15
16 71 original positions by host rapid cytoplasmic streaming. This algal reattachment was
17
18 72 inhibited when host cytoplasmic streaming was arrested by nocodazole. In nocodazole-
19
20
21 73 treated cells, approximately 5 h was required for complete algal recovery, and the host
22
23
24 74 cytoplasmic streaming had been resumed at that time. These results demonstrated that
25
26 75 adhesion of the PV beneath the host cell cortex can be repeatedly induced and that host
27
28 76 cytoplasmic streaming facilitates the recovery of algal attachment. However, the
29
30
31 77 mechanism by which the PV attaches beneath the host cell cortex remains unknown. In
32
33
34 78 this study, to investigate the mechanisms of symbiotic algal adhesion, the distribution of
35
36 79 mitochondria and trichocysts during reattachment was examined by
37
38
39 80 immunofluorescence microscopy using monoclonal antibodies against the mitochondria
40
41 81 and trichocysts. Furthermore, mitochondria of the anterior part of the host without
42
43
44 82 symbiotic algae were also observed.

45
46 83

47 48 84 **Materials and methods**

49 50 85 **Organism cultivation**

51
52
53 86 *Paramecium bursaria* strain Yad1g1N cells (syngen B1 or R3, mating type I) harboring
54
55
56 87 the symbiotic *Chlorella variabilis* strain 1N were used (Kodama and Fujishima 2009,
57
58
59 88 2011). *Paramecium* cells were cultivated in red pea (*Pisum sativum*) extract culture
60

1
2
3
4
5
6 89 medium (Tsukii *et al.* 1995) with a modified Dryl's solution (Dryl 1959; KH_2PO_4 was
7
8 90 used instead of $\text{NaH}_2\text{PO}_4 \cdot 2\text{H}_2\text{O}$) and inoculated with *Klebsiella aerogenes* (ATCC
9
10 91 35028) one day before use. The cultures were in the early stationary phase of growth
11
12 92 one day after the final feeding. All cells used in this study were in this phase.
13
14 93 Cultivation was performed in test tubes (18 mm \times 180 mm) at $25 \pm 1^\circ\text{C}$ under
15
16 94 fluorescent lighting at 20–30 $\mu\text{mol photons m}^{-2}\text{s}^{-1}$ using an incandescent lamp.
17
18
19
20
21
22

23 96 **Transmission electron microscopy (TEM)**

24
25
26 97 Algae-bearing *P. bursaria* were pre-fixed with 2% glutaraldehyde and prepared for
27
28 98 TEM, as described previously (Kodama *et al.* 2011). The paramecia embedded in
29
30 99 Spurr's resin (1969) were sectioned (70 nm thickness) using an ultramicrotome
31
32
33 100 (Reichert Ultracut S; Leica Microsystems, Vienna, Austria) with a diamond knife,
34
35 101 mounted on nickel mesh grids, and stained with lead citrate (Reynolds 1963). The
36
37 102 sections were observed using TEM (CM120; Philips) at 80 kV.
38
39
40

41 103

42 104 **High-speed centrifugation of *P. bursaria* cells**

43
44
45 105 Algae-bearing *P. bursaria* cells were centrifuged at high speed (Kodama and Fujishima
46
47 106 2011). *P. bursaria* cells at a density of 5×10^3 cells/mL were placed in a 1.5 mL
48
49 107 microcentrifuge tube. The tube was then centrifuged using a fixed-angle rotor at $1000 \times$
50
51 108 *g* for 1 min at $25 \pm 1^\circ\text{C}$ (Model 3740; Kubota Corporation, Tokyo, Japan). Before,
52
53 109 immediately after (approx. 3 min), 10 min, and 30 min after the centrifugation, aliquots
54
55 110 of *Paramecium* cells were air-dried on cover glasses (4.5 mm \times 24 mm) and fixed with
56
57
58
59
60

1
2
3
4
5
6 111 4% (w/v) paraformaldehyde dissolved in phosphate-buffered saline (PBS) (137 mM
7
8 112 NaCl, 2.68 mM KCl, 8.1 mM NaHPO₄·12H₂O, 1.47 mM KH₂PO₄, pH 7.2) for 10 min
9
10
11 113 at 4°C.
12

13
14 114

15 16 115 **Indirect immunofluorescence microscopy**

17
18 116 The cover glasses with the fixed *P. bursaria* cells were washed with PBS containing
19
20
21 117 0.05% (v/v) Tween 20 (PBST) for 10 min at 4°C. The cells on the cover glasses were
22
23 118 treated with a culture supernatant of hybridoma cells containing either monoclonal
24
25
26 119 antibodies mAb5A11E2 against trichocysts of *P. bursaria* (Kodama and Fujishima
27
28 120 2011) or mAb2B8A8H1 against mitochondria of *P. bursaria* (Kodama and Fujishima
29
30
31 121 2022) overnight at 4°C, washed with PBS, and treated with Alexa Fluor 488 conjugated
32
33 122 goat anti-mouse IgG (Molecular Probes Inc., Eugene, OR, USA) diluted 1000-fold with
34
35
36 123 PBS for 2 h at 25°C. The cover glasses were then washed with PBS and observed under
37
38 124 a differential interference contrast microscope and fluorescence microscope (BX53;
39
40
41 125 Olympus Corp., Tokyo, Japan). Images were acquired using an Olympus DP74 system
42
43
44 126 and analyzed using Olympus cellSens Dimension software.
45

46 127

47 48 49 128 **Statistical analysis**

50
51 129 Data were analyzed using the Mann-Whitney U-test in R. Reproducibility of the data
52
53 130 was confirmed by three independent experiments.
54

55
56 131

57 58 132 **Results**

1
2
3
4
5
6 **133 Transmission electron microscopy (TEM) of algae-bearing *P. bursaria***
7

8
9 **134** Algae-bearing paramecia were observed using TEM (Fig. 1). As shown by the
10
11 **135** arrowhead in Fig. 1, we observed mitochondria that attach to the PVs, as shown in
12
13 **136** previous studies (Fujishima and Kodama 2012; Song *et al.* 2017). In contrast, the
14
15 **137** trichosyst membrane did not attach to the PV membrane (black arrow in Fig. 1).
16
17
18

19 **138**

20
21 **139 Effects of high-speed centrifugation on distribution of trichocysts in**
22
23 **140 algae-bearing *P. bursaria* cells**
24

25
26 **141** Figure 2A shows the results obtained by indirect immunofluorescence microscopy after
27
28 **142** high-speed centrifugation of algae-bearing *P. bursaria* cells using monoclonal
29
30 **143** antibodies against trichocysts. Before centrifugation, symbiotic algae were shown
31
32 **144** distributed throughout the host cell by differential interference contrast microscopy, and
33
34 **145** immunofluorescence showed that the trichocysts of the algae-bearing cells were
35
36 **146** localized as a ring surrounding the algae, as shown in a previous study (Kodama and
37
38 **147** Fujishima 2011). Immediately after centrifugation, the symbiotic algae dislocated and
39
40 **148** accumulated in the posterior region of *Paramecium*. As for trichocysts,
41
42 **149** immunofluorescence was hardly observed in the centrifuged cells. When cells are
43
44 **150** stimulated, trichocysts discharge their contents in milliseconds (Adoutte 1988).
45
46
47
48

49
50
51
52 **151** Therefore, most trichocysts are discharged via high-speed centrifugation. In fact, we
53
54 **152** observed many discharged trichocysts around the *Paramecium* cells (Fig. 2B). The
55
56 **153** monoclonal antibodies also distinguished after discharge, coinciding with findings in a
57
58
59
60

1
2
3
4
5
6 154 previous study (Kodama and Fujishima 2011) (Fig. 2B, IM). Ten minutes after
7
8 155 centrifugation, the algae completely localized beneath the host cortex, and few
9
10
11 156 trichocysts were observed by immunofluorescence microscopy. Thirty minutes after
12
13 157 centrifugation, trichocysts were barely observed. In fact, it was previously reported that
14
15 158 when the trichocysts are removed by treatment with lysozyme, regeneration of the
16
17 159 mature trichocysts begins at 3 h, even in the presence of lysozyme (Kodama and
18
19 160 Fujishima 2009). Therefore, it seems that 30 min are not sufficient for the recovery of
20
21 161 trichocysts. These results clearly support previous studies (Kodama and Fujishima
22
23 162 2009) that the symbiotic algae do not need trichocysts to attach beneath the host cell
24
25 163 cortex.

26
27 164 Figure 2C shows the immunofluorescence intensity of *P. bursaria* cells before (x-
28
29 165 bar, T-B), immediately after (x-bar, T-Ia), 10 min (x-bar, T-10 min), and 30 min (x-bar,
30
31 166 T-30 min) after centrifugation. The immunofluorescence intensity of the centrifuged
32
33 167 cells decreased drastically and did not increase until 30 min after centrifugation. These
34
35 168 quantitative data correspond well with the images shown in Fig. 2A.

36
37 169

38 170 **Effects of high-speed centrifugation on distribution of mitochondria in** 39 40 171 **algae-bearing *P. bursaria* cells**

41 172 We examined the effect of high-speed centrifugation on host mitochondria by
42
43 173 immunofluorescence microscopy using the same method described above (Fig. 3).
44
45 174 Before centrifugation, immunofluorescence of mitochondria was observed around the
46
47 175 symbiotic algae. Immediately after centrifugation (approximately 3 min), the symbiotic
48
49
50
51
52
53
54
55
56
57
58
59
60

1
2
3
4
5
6 176 algae were dislocated, as shown above. Immunofluorescence was observed throughout
7
8 177 the cytoplasm. Note that the cortical mitochondrial immunofluorescence became strong
9
10 178 after centrifugation. This phenomenon may be due to better accessibility for
11
12 179 visualization of the labeling after detachment of the PV from the cortex. Ten and 30 min
13
14 180 after centrifugation, symbiotic algae were distributed throughout the cell because of
15
16 181 algal relocation. Immunofluorescence of mitochondria was also observed around the
17
18 182 symbiotic algae. These observations suggest that symbiotic algae do not need
19
20 183 trichocysts for their attachment but are colocalized with mitochondria, as shown by
21
22 184 TEM observations (Fig. 1).

23
24
25
26
27
28 185 Figure 3B shows the immunofluorescence intensity of algae-free *P. bursaria* cells
29
30 186 before (x-bar, M-B), immediately after (x-bar, M-Ia), 10 min (x-bar, M-10 min), and 30
31
32 187 min (x-bar, M-30 min) after centrifugation. There was almost no change in
33
34 188 immunofluorescence intensity, and no significant difference was observed between the
35
36 189 data for each time point. These quantitative data correspond well with the images shown
37
38 190 in Fig. 3A.

39
40
41
42
43 191

44 192 **Mitochondrial distribution of host anterior cortex**

45
46 193 Kodama (2013) investigated the symbiotic algal distribution of 14 strains of *P.*
47
48 194 *bursaria*. As a result, all strains had symbiotic algae at the ventral, dorsal, and posterior
49
50 195 cortex, and some cells did not have symbiotic algae at the anterior cortex. This
51
52 196 phenomenon is not strain-, syngen-, or mating-type specific. Only 35% of the strain
53
54 197 *Yad1g1N* cells, also used in this experiment, had symbiotic algae at their anterior
55
56
57
58
59
60

1
2
3
4
5
6 198 cortex. This paper also reported that the artificial trichocyst-discharge experiments by
7
8 199 the treatment of lysozyme clarified that trichocysts of the anterior cortex are difficult to
9
10
11 200 remove. Although the high-speed centrifugation experiment clearly showed that
12
13
14 201 trichocysts are unnecessary for algal reattachment, trichocyst-discharge difficulty of the
15
16 202 anterior cortex may be related to the restrict enrichment of the large PVs.

17
18 203 On the other hand, host mitochondria remained unchanged in localization by high-
19
20
21 204 speed centrifugation. This indicated that the presence of mitochondria may be related
22
23
24 205 for algal adhesion. To determine whether the reason for the algal difficulty in adhering
25
26 206 to the host anterior cortex is due to the low mitochondrial density in this area, we
27
28
29 207 examined whether there were differences in the number and distribution of
30
31 208 mitochondria between cells with and without symbiotic algae in their anterior part using
32
33
34 209 indirect immunofluorescence microscopy with the monoclonal antibodies against host
35
36 210 mitochondria.

37
38
39 211 Figure 4 shows differential interference contrast microscopy images of *P. bursaria*
40
41 212 cells with (Algae+) and without (Algae-) symbiotic algae in the anterior end and
42
43
44 213 immunofluorescence images of those cells using mitochondrial monoclonal antibodies.
45
46 214 Note that mitochondria are present in the anterior end without symbiotic algae as well
47
48
49 215 as in the anterior end with symbiotic algae. This observation shows that not all areas
50
51 216 with mitochondria had algae, but there was a localization bias within the host cell.

52
53
54 217

55
56 218 **Discussion**
57
58
59
60

1
2
3
4
5
6 219 Localization of endosymbionts near the host cell cortex is a universal phenomenon; the
7
8 220 same phenomenon has been observed in other ciliate -algae or -cyanobacteria
9
10
11 221 endosymbionts, such as *Mayorella viridis*, *Coleps hirtus*, *Coleps spetai*, *Frontonia*
12
13 222 *leucas*, *Malacophrys sphagni*, *Ophrydium versatile*, *Vorticella* sp., *Climacostomum*
14
15
16 223 *virens*, *Euplotes daidaleos*, *Halteria bifurcata*, *Stentor polymorphus*, and *Stentor niger*
17
18 224 (Reisser 1986). Furthermore, the ability to adjust the intracellular symbiont position is
19
20
21 225 likely an important means of optimizing carbon production (Petrou *et al.* 2017).
22

23 226 A previous study showed that the infectivity of *Chlorella* species in *P. bursaria* is
24
25
26 227 based on their ability to localize beneath the host cell membrane after escaping from the
27
28 228 host digestive vacuole during the early infection process (Kodama and Fujishima 2007).
29
30
31 229 Furthermore, this algal attachment may be related to the avoidance of host lysosomal
32
33 230 digestion or fusion because algal digestion when the symbiotic algae are attached
34
35
36 231 beneath the host cell cortex has not been observed (Kodama and Fujishima unpubl.
37
38 232 data).
39

40
41 233 Acidosomes are organelles responsible for the acidification of DVs before
42
43
44 234 lysosomal fusion and are distributed throughout the cell (Allen 1993; Kodama 2013).
45
46 235 High-speed centrifugation can also induce the rapid accumulation of host acidosomes
47
48 236 and lysosomes at the anterior end of *Paramecium*. Within 10 min of centrifugation, the
49
50
51 237 accumulated vesicles recover their original positions by host rapid cytoplasmic
52
53 238 streaming (Kodama 2013). This observation showed that although acidosomes and
54
55
56 239 lysosomes were dislocated by centrifugation, the mitochondria remained unchanged.
57
58
59
60

1
2
3
4
5
6 240 This is probably because acidosomes and lysosomes undergo cytoplasmic streaming,
7
8 241 but mitochondria do not.

9
10
11 242 Figure 5 shows a schematic representation of the algal attachment mechanism
12
13 243 beneath the host cell cortex after dislocation via high-speed centrifugation. After the
14
15 244 high-speed centrifugation, almost all trichocysts are released out of the cell. The
16
17 245 discharged trichocysts were regenerate from the endoplasmic reticulum, pass through
18
19 246 the Golgi apparatus, and undergo a maturation process (pretrichocysts) before mature
20
21 247 trichocysts are delivered to the cell membrane (Plattner 2017). Pretrichocysts contain a
22
23 248 growing mass of electron dense secretory material. The pretrichocysts then elongate
24
25 249 while their luminal space is progressively filled with crystallizing secretory materials
26
27 250 (Garreau De Loubresse 1993). Because several hours are required for regeneration after
28
29 251 trichocyst discharge (Harumoto 2002), no trichocysts are observed in the cells after
30
31 252 algal reattachment. This representation can be defined by obtaining monoclonal
32
33 253 antibodies against both mitochondria and trichocysts. To the best of our knowledge, this
34
35 254 is the first report showing the distribution of host mitochondria and trichocysts during
36
37 255 algal relocation beneath the host cell cortex.

38
39 256 Fast cytoplasmic streaming after high-speed centrifugation of *P. bursaria* is the
40
41 257 driving force for algal relocation. The characteristic feature of rotational cytoplasmic
42
43 258 streaming in the genus *Paramecium* (*P. aurelia*, *P. caudatum*, *P. bursaria*, *P.*
44
45 259 *multimicronucleatum*, and *P. calkinsi*) is that the pattern of its route and direction
46
47 260 remain constant (Sikora 1981). Various factors, such as cell division, conjugation,
48
49 261 temperature, and osmotic pressure, are known to affect cytoplasmic streaming in
50
51
52
53
54
55
56
57
58
59
60

1
2
3
4
5
6 262 *Paramecium* (Sikora 1981), but the detachment of the symbiotic algae by high-speed
7
8 263 centrifugation is also a factor. In *P. bursaria*–*Chlorella* endosymbiosis, their cell cycle
9
10 264 pace is guaranteed to be synchronous (Takahashi 2016). Takahashi *et al.* (2007) found
11
12 265 that *P. bursaria* controls the proliferation of endosymbiotic algae through host-cell-
13
14 266 cycle-dependent cytoplasmic streaming. Thus, host cytoplasmic streaming plays a major
15
16 267 role in maintaining the number and cellular localization of symbiotic algae in *P.*
17
18
19
20
21 268 *bursaria* cells.

22
23 269 The mitochondria of the host *P. bursaria* are close to the PV membrane (Fig. 1),
24
25 270 and their number and function are altered by the presence or absence of symbiotic algae
26
27 271 (Kodama and Fujishima 2022). Therefore, host mitochondria and symbiotic algae are
28
29 272 expected to be strongly associated with each other. We assumed that the presence of
30
31 273 mitochondria was essential for the existence of symbiotic algae; however, the results in
32
33 274 Fig. 4 contradict this expectation. Mitochondria and endoplasmic reticulum (ER) are
34
35 275 contact at ER-Mitochondria Contact Sites (EMCS)s. Lipids and Ca²⁺ synthesized in the
36
37 276 ER are transported to mitochondria through the EMCSs (Kornmann *et al.* 2009; Rizzuto
38
39 277 *et al.* 1998). Furthermore, EMCs is also important in reactive oxygen species (ROS)
40
41 278 production (Booth *et al.* 2016) and autophagosome formation (Hamasaki *et al.* 2013).
42
43 279 Song *et al.* (2017) reported that the host ER is also involved in organizing intracellular
44
45 280 algal symbiosis in the cytoplasm. The relationship between host mitochondria and
46
47 281 symbiotic algae and the relationship between other cellular organelles, including the
48
49 282 host endoplasmic reticulum, should be examined in detail in future studies.
50
51
52
53
54
55
56
57
58
59
60

1
2
3
4
5
6 283 The exposure of *P. bursaria* cells to ultraviolet and photosynthetically active
7
8 284 radiation is known to induce symbiotic algal dislocation, moving to the posterior cell
9
10 285 region (Summerer *et al.* 2009). The displaced algae relocate to a "normal" distribution
11
12 286 when host cells are transferred to a medium without ultraviolet radiation conditions. The
13
14 287 mechanisms of algal displacement and relocation remain unclear (Summerer *et al.*
15
16 288 2009). A dislocation of *Chlorella* symbionts has also been described in the ciliate
17
18 289 *Pelagodileptus trachelioides* (Butkay 2004). Although this phenomenon appears to be a
19
20 290 stress reaction followed by host cytolysis, the details remain unknown. The method of
21
22 291 artificial algal detachment and subsequent rapid reattachment by high-speed
23
24 292 centrifugation of algae-bearing *P. bursaria* may contribute to understanding the
25
26 293 mechanism of symbiotic algal localization in a variety of host species.

27
28 294 Although the mechanism of PV localization beneath the host cell cortex is still
29
30 295 unknown, the docking or distribution mechanism of mitochondria and uninserted
31
32 296 trichocysts in other ciliate species, such as *Paramecium tetraurelia*, have been reported.
33
34 297 Aufderheide (1977) reported that, in the subcortical regions of *P. tetraurelia*,
35
36 298 mitochondria and uninserted (undocked) trichocysts display saltatory motility with
37
38 299 individual characteristics, making them distinguishable from each other and from
39
40 300 cellular cyclosis. The saltatory motion of trichocysts is implicated as a means of
41
42 301 transporting new trichocysts from the cytoplasm to their ultimate location in the cellular
43
44 302 cortex. In addition, the saltatory motion may be a factor in the intracellular distribution
45
46 303 of the mitochondria. The mechanism of trichocyst docking at the *Paramecium* cell
47
48 304 membrane has been reported in detail (Plattner 2017; Plattner *et al.* 1982). Before
49
50
51
52
53
54
55
56
57
58
59
60

1
2
3
4
5
6 305 trichocyst docking, mature trichocysts move through the subcortical region by cyclosis
7
8 306 (Sikora 1981). Then, they approach, in a saltatory manner, tip first, a docking site at the
9
10 307 cell membrane (Aufderheide 1978); Thereby, trichocysts are guided by the same
11
12 308 “hand.” This entity is a microtubule emanating from a nearby ciliary basal body
13
14 309 (Plattner *et al.* 1982), which always is situated ~ 0.5 to 1 µm from a docking site proper.
15
16 310 Petrou *et al.* (2017) found that the intracellular algal symbionts within the large
17
18 311 photosymbiotic foraminifera *Marginopora vertebralis* exhibit phototactic behavior and
19
20 312 the phototactic movement of the symbionts is accomplished by the host through rapid
21
22 313 actin-mediated relocation of the symbionts deeper into the cavities within the calcite
23
24 314 skeletons. Thus, the host cytoskeleton plays a major role in the migration of host
25
26 315 cytosolic organelles and symbiotic algae. In order to study the involvement of the
27
28 316 cytoskeleton in the process of PV localization, the cell cortex of algae-bearing *P.*
29
30 317 *bursaria* cells was observed in detail by TEM. However, no cytoskeletal structure that
31
32 318 seems to be related to PV localization has been found until now (Kodama unpub. Data).
33
34 319 Adhesion just below the host cell surface is a universal phenomenon found not
35
36 320 only in cell organelles and symbiotic algae but also in pathogenic bacteria. Protists are
37
38 321 potential natural hosts of various bacterial species, including pathogens. *Paramecium*
39
40 322 *caudatum* could be a natural reservoir of *Legionella pneumophila* (Watanabe *et al.*
41
42 323 2016), and *P. bursaria* could be a potential host of *Francisella novicida* (Watanabe *et*
43
44 324 *al.* 2022). *Francisella novicida* is a facultative intracellular pathogen and a causative
45
46 325 agent of tularemia (Ellis *et al.* 2002). Recently, Watanabe *et al.* (2022) found that *F.*
47
48 326 *novicida* (strain U112) cells, which were wrapped with the host DV membrane,
49
50 327 localized beneath the host cell cortex. This indicates that both symbiotic algae and
51
52
53
54
55
56
57
58
59
60

1
2
3
4
5 328 bacteria can attach beneath the host cell cortex. Therefore, elucidation of the mechanism
6
7 329 of localization beneath the host cell cortex using *P. bursaria* is expected to contribute to
8
9 330 the development of not only symbiotic biology but also infectious disease research.
10
11

12 331

13 332 **References**

14
15
16
17 333 Adoutte A. Exocytosis: Biogenesis, Transport and Secretion of Trichocysts. In: Görtz
18
19 334 HD (ed). *Paramecium*. Springer: Berlin, Heidelberg, 1988, 325–62.
20
21

22 335

23
24 336 Allen RD. Acidosomes: recipients of multiple sources of membrane and cargo during
25
26 337 development and maturation. *J Cell Sci* 1993;**106**:411–22.
27
28

29 338

30
31 339 Albers D, Wiessner W. Nitrogen nutrition of endosymbiotic *Chlorella* spec. *Endocyt*
32
33 340 *Cell Res* 1985;**1**:55–64.
34
35

36 341

37
38 342 Albers D, Reisser W, Wiessner W. Studies on the nitrogen supply of endosymbiotic
39
40 343 chlorellae in green *Paramecium bursaria*. *Plant Sci Lett* 1982;**25**:85–90.
41
42

43 344

44
45 345 Aufderheide KJ. Saltatory motility of uninserted trichocysts and mitochondria in
46
47 346 *Paramecium tetraurelia*. *Science* 1977;**198**:299–300.
48
49

50 347

51
52 348 Aufderheide KJ. Motility events of trichocyst insertion in *Paramecium tetraurelia*. *J*
53
54 349 *Protozool* 1978;**25**:362–65.
55
56

57 350
58
59
60

- 1
2
3
4
5 351 Blanc G, Duncan G, Agarkova I *et al.* The *Chlorella variabilis* NC64A genome reveals
6
7 352 adaptation to photosynthesis, coevolution with viruses, and cryptic sex. *The Plant Cell*
8
9 353 2010;**22**:2943–55.
10
11
12 354
13
14 355 Booth DM, Enyedi B, Geiszt M *et al.* Redox Nanodomains Are Induced by and Control
15
16 356 Calcium Signaling at the ER-Mitochondrial Interface. *Mol Cell* 2016;**63**:240–48.
17
18
19 357
20
21 358 Brown JA, Nielsen PJ. Transfer of photosynthetically produced carbohydrate from
22
23 359 endosymbiotic *Chlorellae* to *Paramecium bursaria*. *J Protozool* 1974;**21**:569–70.
24
25
26 360
27
28 361 Butkay M. Beobachtungen an *Pelagodileptus trachelioides* (Ciliophora). *Lauterbornia*
29
30 362 2004;**49**:129–40.
31
32
33 363
34
35 364 Cheng YH, Liu CFJ, Yu YH *et al.* Genome plasticity in *Paramecium bursaria* revealed
36
37 365 by population genomics. *BMC Biology* 2020;**18**:180.
38
39
40 366
41
42 367 Dryl S. Antigensic transformation in *Paramecium aurelia* after homologous antiserum
43
44 368 treatment during autogamy and conjugation. *J Protozool* 1959;**6**:25.
45
46
47 369
48
49 370 Ellis J, Oyston PC, Green M *et al.* Tularemia. *Clin Microbiol Rev* 2002;**15**:631–46.
50
51 371
52
53 372 Fujishima M, Kodama Y. Endosymbionts in *Paramecium*. *Europ J Protistol*
54
55 373 2012;**48**:124–37.
56
57
58 374
59
60

- 1
2
3
4
5 375 Garreau De Loubresse N. Early steps of the secretory pathway in *Paramecium*:
6
7 376 ultrastructural, immunocytochemical, and genetic analysis of trichocyst biogenesis. In:
8
9 377 Plattner H (ed). *Membrane Traffic in Protozoa*, JAI Press, Greenwich (CT, USA),
10
11 378 London (GB), 1993, 27–59.
12
13
14 379
15
16 380 Gortz HD. Infections of *Paramecium bursaria* with bacteria and yeasts. *J Cell Sci* 1982;
17
18 381 58:445–53.
19
20 382
21
22 383 Gu FK, Chen L, Ni B, Zhang X. A comparative study on the electron microscopic
23
24 384 enzymo-cytochemistry of *Paramecium bursaria* from light and dark cultures. *Eur J*
25
26 385 *Protistol* 2002;38:267–78.
27
28 386
29
30 387 Hamasaki M, Furuta N, Matsuda A *et al.* Autophagosomes form at ER-mitochondria
31
32 388 contact sites. *Nature* 2013;495:389–93.
33
34 389
35
36 390 Harumoto T. Defensive function of trichocysts in *Paramecium*. *Jpn J Protozool*
37
38 391 2002;35:125–33.
39
40 392
41
42 393 Karakashian SJ, Rudzinska MA. Inhibition of lysosomal fusion with symbiont-
43
44 394 containing vacuoles in *Paramecium bursaria*. *Exp Cell Res* 1981;131:387–93.
45
46 395
47
48 396 Kodama Y. Localization of attachment area of the symbiotic *Chlorella variabilis* of the
49
50 397 ciliate *Paramecium bursaria* during the algal removal and reinfection. *Symbiosis*
51
52 398 2013;60:25–36.
53
54
55
56
57
58
59
60

- 1
2
3
4
5 399 Kodama Y, Fujishima M. Infectivity of *Chlorella* species for the ciliate *Paramecium*
6
7 400 *bursaria* is not based on sugar residues of their cell wall components, but on their
8
9 401 ability to localize beneath the host cell membrane after escaping from the host digestive
10
11 402 vacuole in the early infection process. *Protoplasma* 2007;**231**:55–63.
12
13
14 403
15
16 404 Kodama Y, Fujishima M. Localization of perialgal vacuoles beneath the host cell
17
18 405 surface is not a prerequisite phenomenon for protection from the host's lysosomal fusion
19
20 406 in the ciliate *Paramecium bursaria*. *Protist* 2009;**160**:319–29.
21
22
23 407
24
25 408 Kodama Y, Fujishima M. Secondary symbiosis between *Paramecium* and *Chlorella*
26
27 409 cells. *Int Rev Cell Mol Biol* 2010;**279**:33–77.
28
29 410
30
31 411 Kodama Y, Fujishima M. Endosymbiosis of *Chlorella* species to the ciliate
32
33 412 *Paramecium bursaria* alters the distribution of the host's trichocysts beneath the host
34
35 413 cell cortex. *Protoplasma* 2011;**248**:325–37.
36
37 414
38
39 415 Kodama Y, Fujishima M. Synchronous induction of detachment and reattachment of
40
41 416 symbiotic *Chlorella* spp. from the cell cortex of the host *Paramecium bursaria*. *Protist*
42
43 417 2013;**164**:660–72.
44
45 418
46
47 419 Kodama Y, Fujishima M. Endosymbiotic *Chlorella variabilis* reduces mitochondrial
48
49 420 number in the ciliate *Paramecium bursaria*. *Sci Rep* 2022;**12**:8216.
50
51 421
52
53
54
55
56
57
58
59
60

- 1
2
3
4
5 422 Kodama Y, Inouye I, Fujishima M. Symbiotic *Chlorella vulgaris* of the ciliate
6
7 423 *Paramecium bursaria* plays an important role in maintaining perialgal vacuole
8
9 424 membrane functions. *Protist* 2011;**162**:288–303.
10
11
12 425
13
14 426 Kornmann B, Currie E, Collins SR *et al.* An ER-mitochondria tethering complex
15
16 427 revealed by a synthetic biology screen. *Science* 2009;**325**:477–81.
17
18
19 428
20
21 429 Petrou K, Ralph PJ, Nielsen DA. A novel mechanism for host-mediated photoprotection
22
23 430 in endosymbiotic foraminifera. *ISME J* 2017;**11**:453–62.
24
25
26 431
27
28 432 Plattner H. Trichocysts-*Paramecium*'s Projectile-like Secretory Organelles: Reappraisal
29
30 433 of their Biogenesis, Composition, Intracellular Transport, and Possible Functions. *J*
31
32 434 *Eukaryot Microbiol* 2017;**64**:106–33.
33
34
35 435
36
37 436 Plattner H, Westphal C, Tiggemann R. Cytoskeleton secretory vesicle interactions
38
39 437 during the docking of secretory vesicles at the cell membrane in *Paramecium*
40
41 438 *tetraurelia* cells. *J Cell Biol* 1982;**92**:368–77.
42
43
44 439
45
46 440 Reisser W. Endosymbiotic associations of freshwater protozoa and algae. In: Corliss
47
48 441 JO, Patterson DJ (eds). *Progress in Protistology*, Biopress Ltd, Bristol, 1986, 195–214.
49
50
51 442
52
53 443 Rizzuto R, Pinton P, Carrington W *et al.* Close contacts with the endoplasmic reticulum
54
55 444 as determinants of mitochondrial Ca²⁺ responses. *Science* 1998;**280**:1763–66.
56
57
58 445
59
60

- 1
2
3
4
5 446 Sikora J. Cytoplasmic streaming in *Paramecium*. *Protoplasma* 1981;**109**:57–77.
6
7 447
8
9 448 Song C, Murata K, Suzuki T. Intracellular symbiosis of algae with possible involvement
10
11 449 of mitochondrial dynamics. *Sci Rep* 2017;**7**:1221.
12
13 450
14
15 451 Summerer M, Sonntag B, Hörtnagl P *et al.* Symbiotic ciliates receive protection against
16
17 452 UV damage from their algae: A test with *Paramecium bursaria* and *Chlorella*. *Protist*
18
19 453 2009;**160**:233–43.
20
21 454
22
23 455 Takahashi T. Simultaneous Evaluation of Life Cycle Dynamics between a Host
24
25 456 *Paramecium* and the Endosymbionts of *Paramecium bursaria* Using Capillary Flow
26
27 457 Cytometry. *Sci Rep* 2016;**6**:31638.
28
29 458
30
31 459 Takahashi T, Shirai Y, Kosaka T *et al.* Arrest of Cytoplasmic Streaming Induces Algal
32
33 460 Proliferation in Green *Paramecia*. *PLoS ONE* 2007;**12**:e1352.
34
35 461
36
37 462 Tsukii Y, Harumoto T, Yazaki K. Evidence for a viral macro- nuclear endosymbiont in
38
39 463 *Paramecium caudatum*. *J Euk Microbiol* 1995;**42**:109–15.
40
41 464
42
43 465 Reisser W. The metabolic interactions between *Paramecium bursaria* Ehrbg. and
44
45 466 *Chlorella spec.* in the *Paramecium bursaria*-symbiosis. II. Symbiosis-specific
46
47 467 properties of the physiology and the cytology of the symbiotic unit and their regulation
48
49 468 (author's transl). *Arch Microbiol* 1976;**111**:161–70.
50
51 469
52
53
54
55
56
57
58
59
60

- 1
2
3
4
5 470 Reisser W. The metabolic interactions between *Paramecium bursaria* Ehrbg. and
6
7 471 *Chlorella* spec. in the *Paramecium bursaria*-symbiosis. III. The influence of different
8
9 472 CO₂-concentrations and of glucose on the photosynthetic and respiratory of the
10
11 473 symbiotic unit. *Arch Microbiol* 1980;**125**:291–93.
12
13 474
14
15 475 Reisser W. Endosymbiotic associations of freshwater protozoa and algae. *Prog Protistol*
16
17 476 1986;**1**:195–214.
18
19 477
20
21 478 Reynolds ES. The use of lead citrate at high pH as an electron-opaque stain in electron
22
23 479 microscopy. *J Cell Biol* 1963;**17**:208–12.
24
25 480
26
27 481 Spurr AR. A low viscosity epoxy resin embedding medium for electron microscopy. *J*
28
29 482 *Ultrastruct Res* 1969;**26**:31–43.
30
31 483
32
33 484 Watanabe K, Nakao R, Fujishima M *et al.* Ciliate *Paramecium* is a natural reservoir of
34
35 485 *Legionella pneumophila*. *Sci Rep* 2016;**6**:24322.
36
37 486
38
39 487 Watanabe K, Motonaga A, Tachibana M *et al.* *Francisella novicida* can
40
41 488 utilize *Paramecium bursaria* as its potential host. *Envl Microbiol Rep* 2022;**14**:50–9.
42
43
44
45
46
47
48
49
50
51
52
53
54
55
56
57
58
59
60

Figure Legends

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

Figure 2. (A) Indirect immunofluorescence micrographs of the high-speed centrifuged algae-bearing *Paramecia* with the monoclonal antibody against trichocysts. Before centrifugation, the symbiotic algae localized throughout the host cell were observed by differential interference contrast (DIC) microscopy. Immunofluorescence (IM) is observed in the whole cell.

Immediately after the centrifugation, the symbiotic algae were dislocated and concentrated at the posterioranterior sideside. Ten min after the centrifugation, the dislocated algae distributed throughout the cell by host cytoplasmic streaming. Thirty min after the centrifugation, algal re-localization was completed. IM is hardly observed in the centrifuged cell because of discharge of trichocysts induced by the high-speed centrifugation as shown in (B). Immunofluorescence is still not observed even though the algal re-localized. Ma, macronucleus. **(B) DIC and IM micrographs of discharged trichocysts from a certain *P. bursaria* cell. The trichocyst is completely free from the *P. bursaria*. Trichocysts are colorless and transparent, making them difficult to observe by the DIC microscopy, but easy to observe by the IM.** (C)

Immunofluorescence intensity of the centrifuged cells decreased drastically, and did not increase. ~~Twelve to 20 *Paramecium* cells were observed.~~ Error bars show standard deviation (SD). Asterisks indicate significant differences (Mann-Whitney U test, ***P < 0.001, Twelve to 20 *Paramecium* cells were observed).

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

1
2
3 reattachment was complete within 30 minutes and no change in mitochondrial fluorescence was
4 observed. Immediately after the high-speed centrifugation, the symbiotic algae localized to the
5 posterior side. Thus, only the posterior side of the *Paramecia* becomes thicker and stiffer due to
6 the presence of many algae, making it really difficult to focus on the posterior side. Therefore,
7 the focus of the immediate after is different from that of the other three (Before, 10 min, and 30
8 min) periods. Ma, macronucleus. (B) Immunofluorescence intensity of the centrifuged cells did
9 not change before and after the centrifugation, and there was no significant differences (Mann-
10 Whitney U test, Ten to 12 *Paramecium* cells were observed) ~~were observed. Ten to 12~~
11 ~~*Paramecium* cells were observed.~~ Error bars show standard deviation (SD).
12
13
14
15
16
17
18
19

20
21 **Figure 4.** Indirect immunofluorescence micrographs with the monoclonal antibody against
22 mitochondria. *Paramecium bursaria* cells with (Algae+) and without (Algae-) symbiotic algae
23 on their anterior were observed by differential interference contrast (DIC) microscopy.
24 Immunofluorescence (IM) indicates that host mitochondria is present in areas with and without
25 symbiotic algae.
26
27
28
29
30

31 **Figure 5.** A schematic diagram of changes in localization of symbiotic algae with PV membrane,
32 host mitochondria, and trichocysts before and after high-speed centrifugation. Before the
33 centrifugation, numerous host mitochondria and trichocysts are attached beneath the host cell
34 cortex, with symbiotic algae settled between them. After the centrifugation, the symbiotic algae
35 detach from the host cell cortex, immediately accumulating at the posterior end of the host cell.
36 At the same time, almost all trichocysts are released out of the cell, but mitochondria remain
37 anchored. The symbiotic algae gradually reattach by host cytoplasmic streaming, which is
38 complete within 30 min. At that time, the algae attach between mitochondria, but trichocysts are
39 not required for the algal attachment.
40
41
42
43
44
45
46
47
48
49
50
51
52
53
54
55
56
57
58
59
60

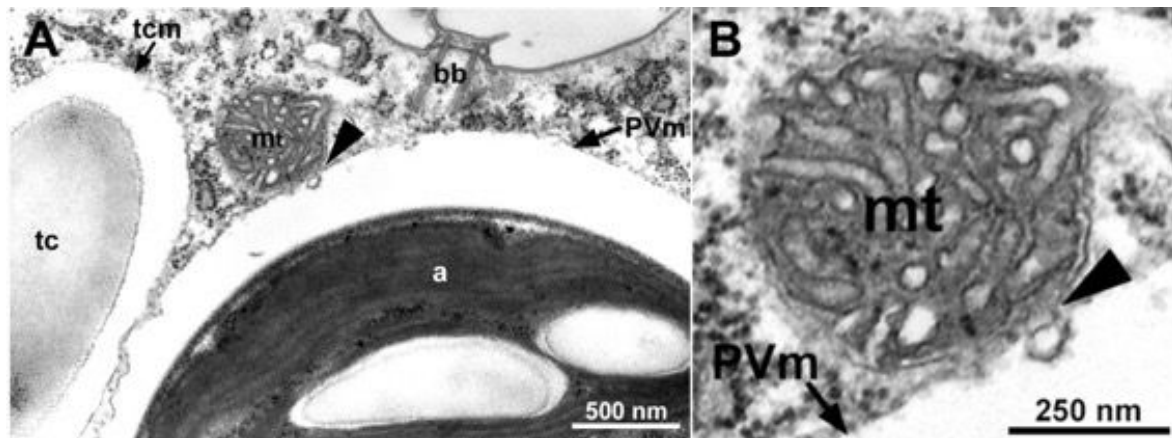


Figure 1. (A) Transmission electron microscopy of the symbiotic algae and the surrounding trichocyst and mitochondria. The magnified image of the mitochondria is shown in (B). Note that mitochondrion is attached to a PV membrane (PVm, arrowhead). tc, trichocyst; tcm, trichocyst membrane; mt, mitochondrion;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

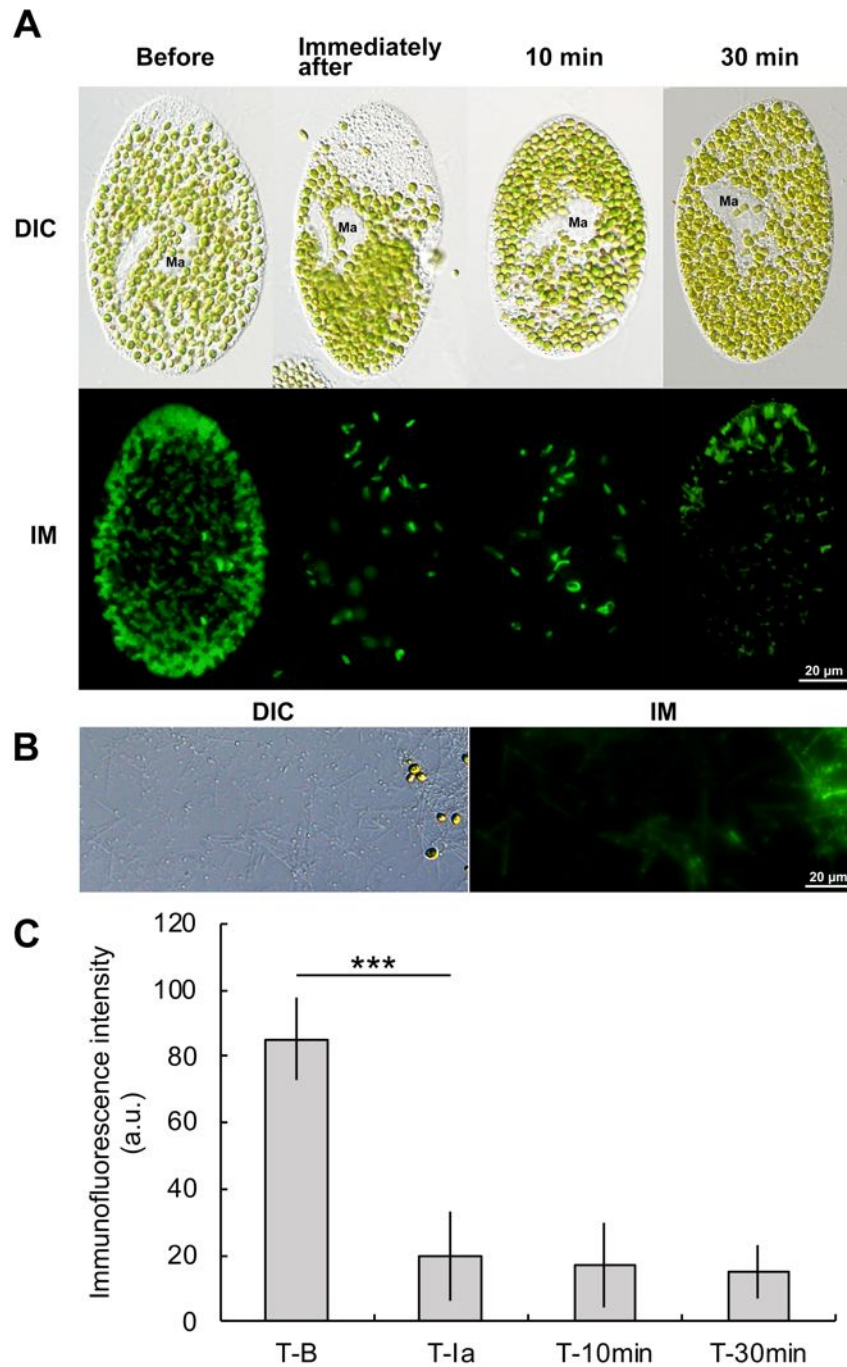


Figure 2. (A) Indirect immunofluorescence micrographs of the high-speed centrifuged algae-bearing *Paramecia* with the monoclonal antibody against trichocysts. Before centrifugation, the symbiotic algae localized throughout the host cell were observed by differential interference contrast (DIC) microscopy. Immunofluorescence (IM) is observed in the whole cell. Immediately after the centrifugation, the symbiotic algae were dislocated and concentrated at the

1
2
3 anterior-posterior side. Ten min after the centrifugation, the dislocated algae distributed
4 throughout the cell by host cytoplasmic streaming. Thirty min after the centrifugation, algal re-
5 localization was completed. IM is hardly observed in the centrifuged cell because of discharge of
6 trichocysts induced by the high-speed centrifugation (**B**). Immunofluorescence is still not
7 observed even though the algal re-localized. Ma, macronucleus. (**C**) Immunofluorescence
8 intensity of the centrifuged cells decreased drastically, and did not increase. ~~Twelve to 20~~
9 ~~Paramecium cells were observed~~. Error bars show standard deviation (SD). Asterisks indicate
10 significant differences (Mann-Whitney U test, ***P < 0.001, Twelve to 20 Paramecium cells
11 were observed).
12
13
14
15
16
17
18
19
20
21
22
23
24
25
26
27
28
29
30
31
32
33
34
35
36
37
38
39
40
41
42
43
44
45
46
47
48
49
50
51
52
53
54
55
56
57
58
59
60

For Peer Review

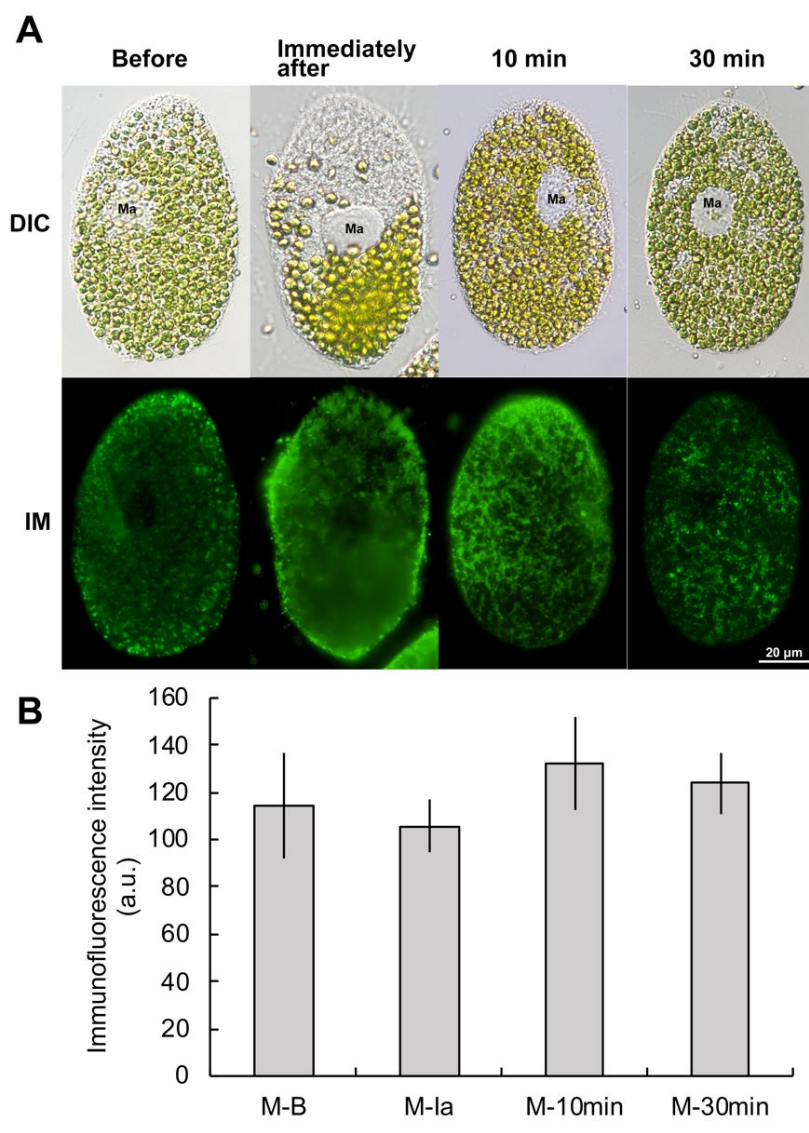


Figure 3. (A) Indirect immunofluorescence micrographs of the high-speed centrifuged algae-bearing *Paramecia* with the monoclonal antibody against mitochondria.

Photographs were taken by focusing on mitochondria showing strong immunofluorescence during each observation period. Before the centrifugation, the symbiotic algae were observed throughout the host cell were observed by differential interference contrast (DIC) microscopy. Immunofluorescence (IM) is also observed in the whole cell. Immediately after the high-speed centrifugation, the symbiotic algae localized to

1
2
3
4
5
6 the posterior side. Thus, only the posterior side of the *Paramecia* becomes thicker and stiffer due to
7 the presence of many algae, making it really difficult to focus on the posterior side. Therefore, the
8 focus of the immediate after is different from that of the other three (Before, 10 min, and 30 min)
9 periods. Ma, macronucleus. **(B)** Immunofluorescence intensity of the centrifuged cells
10
11 did not change before and after the centrifugation, and no significant differences (Mann-
12
13 Whitney U test, ten to 12 *Paramecium* cells were observed) were observed. Error bars
14
15 show standard deviation (SD).
16
17
18
19
20
21
22
23
24
25
26
27
28
29
30
31
32
33
34
35
36
37
38
39
40
41
42
43
44
45
46
47
48
49
50
51
52
53
54
55
56
57
58
59
60

For Peer Review

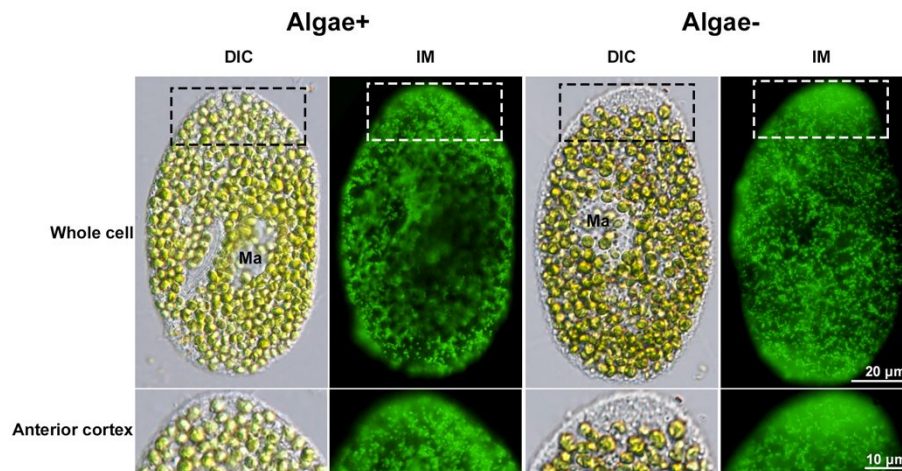


Figure 4. Indirect immunofluorescence micrographs with the monoclonal antibody against mitochondria. *Paramecium bursaria* cells with (Algae+) and without (Algae-) symbiotic algae on their anterior were observed by differential interference contrast (DIC) microscopy. Immunofluorescence (IM) indicates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

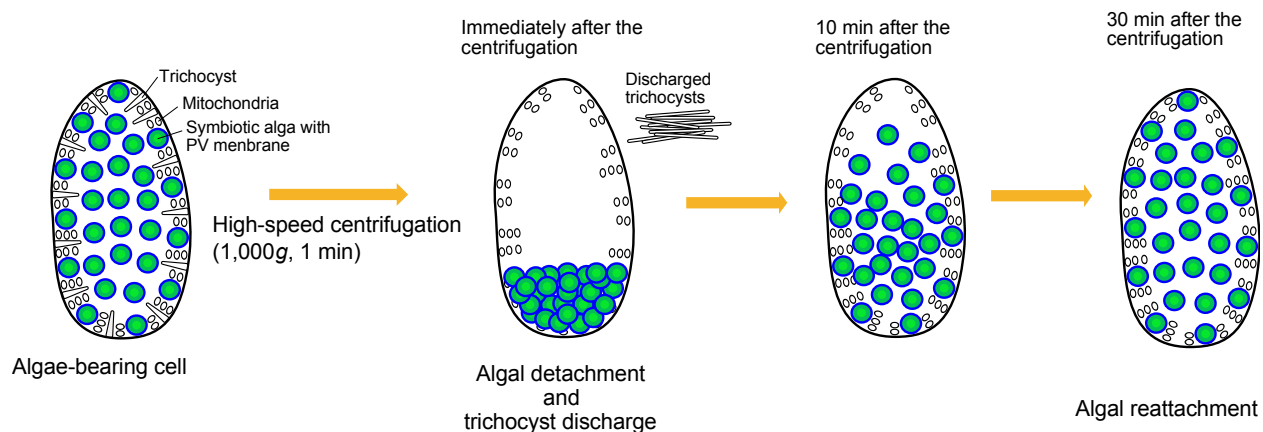


Figure 5. A schematic diagram of changes in localization of symbiotic algae with PV membrane, host mitochondria, and trichocysts before and after high-speed centrifugation. Before the centrifugation, numerous host mitochondria and trichocysts are attached beneath the host cell cortex, with symbiotic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

1
2
3
4
5
6 1 **Role of host ciliate *Paramecium bursaria* mitochondria and trichocysts for**
7
8 2 **symbiotic *Chlorella variabilis* attachment beneath the host cell cortex**
9

10
11 3
12
13 4 Mitochondria and trichocysts of the ciliate *Paramecium bursaria*
14
15
16 5

17
18 6 **One sentence summary:** Although the host mitochondria of ciliate *Paramecium*
19
20 7 *bursaria* are near symbiotic algae during reattachment, this does not mean that the algae
21
22 8 can exist if mitochondria are present, indicating some localization bias within the host
23
24 9 cell.
25
26
27
28
29
30

31 11 **Abstract**
32

33 12 Symbiotic *Chlorella variabilis* is encased in the perialgal vacuole (PV) membrane of
34
35 13 ciliate *Paramecium bursaria*. The PV membrane is stably anchored below the host cell
36
37 14 cortex by adhesion to host mitochondria. Host trichocysts, which are defensive
38
39 15 organelles against predators, are present in the mitochondria and PV membrane vicinity.
40
41 16 The mechanism by which PV attaches beneath the host cell cortex remains unknown.
42
43 17 When *P. bursaria* is centrifuged at high speed, the symbiotic algae are displaced from
44
45 18 the host cell cortex and concentrate at the posterior end. When centrifugation is stopped,
46
47 19 the dislocated algae reattach beneath the host cell cortex with fast cytoplasmic
48
49 20 streaming. The densities of mitochondria and trichocysts before and after centrifugation
50
51 21 were compared using indirect immunofluorescence microscopy with monoclonal
52
53 22 antibodies. Almost all trichocysts were shed by high-speed centrifugation, but
54
55
56
57
58
59
60

1
2
3
4
5
6 23 dislocated algae could reattach even in the absence of trichocysts. In contrast, host
7
8 24 mitochondria were unaffected in localization and number, and the dislocated algae also
9
10
11 25 reattached. These findings suggest trichocysts are unnecessary for algal re-localization_
12
13
14 26 and that mitochondria are colocalized with the algae, unlike mitochondria. However,
15
16 27 many mitochondria were also present in the cell's anterior region without symbiotic
17
18 28 algae. Therefore, not all areas with mitochondria contained algae, but there was a
19
20
21 29 localization bias within the host cell.
22
23
24 30

25
26 31 **Keywords:** Algal reattachment, *Chlorella variabilis*, Endosymbiosis, Mitochondria,
27
28 32 *Paramecium bursaria*, Trichocyst
29
30
31 33

32 34 Introduction

33
34 35 *Paramecium bursaria*, a freshwater ciliate, is a symbiotic organism that establishes
35
36 36 endosymbiotic relationships with *Chlorella* spp. Each symbiotic alga is enclosed in a
37
38 37 perialgal vacuole (PV) derived from the host digestive vacuole (DV), which protects the
39
40 38 alga from lysosomal fusion (Gu *et al.* 2002; Karakashian and Rudzinska 1981). This
41
42 39 relationship between ciliates and algae is mutualistic; the host cell supplies algae with
43
44 40 nitrogen and oxygen (Albers *et al.* 1982, 1985; Reisser 1976, 1980), whereas the algae
45
46 41 provide the host with photosynthetic products (Brown *et al.* 1974; Reisser 1986) and
47
48 42 CO₂ (Reisser 1980). However, both *P. bursaria* and symbiotic algae can live without
49
50 43 their partners. The re-establishment of endosymbiosis between algae-free (removed) *P.*
51
52 44 *bursaria* cells and symbiotic *Chlorella* cells isolated from algae-bearing hosts can be
53
54
55
56
57
58
59
60

1
2
3
4
5
6 45 induced (Kodama and Fujishima 2010). In addition to their original symbiotic algae,
7
8 46 algae-free *P. bursaria* cells can be reinfected with bacteria and yeast that are retained in
9
10
11 47 the cytoplasm (Görtz 1982; Watanabe *et al.* 2022). The nuclear genomes of the
12
13 48 symbiotic *Chlorella variabilis* (Blanc *et al.* 2010) and the host *P. bursaria* have been
14
15
16 49 explored (Cheng *et al.* 2020); these organisms are now considered models for
17
18
19 50 endosymbiosis research.

20
21 51 Using pulse-labeling and chasing of algae-free paramecia for 1.5 min with
22
23 52 symbiotic algae that were isolated from algae-bearing *P. bursaria*, four important
24
25
26 53 cytological events have been identified. These events are necessary for the
27
28
29 54 establishment of endosymbiosis and the timing of each mechanism during the infection
30
31 55 process (Kodama and Fujishima 2010). 1) Within 3 min of algal mixing, part of the
32
33 56 algae has resistance to the host's lysosomal digestive enzymes in the DVs. 2) Within 30
34
35
36 57 min of mixing, algae in the DV begin budding from the DV membrane into the
37
38
39 58 cytoplasm. 3) Within 15 min of budding from the DV, the DV membrane enclosing a
40
41 59 single green *Chlorella* differentiates into a PV membrane. 4) The PV membrane
42
43
44 60 translocates beneath the host cell cortex.

45
46 61 The PV appears to localize near the host mitochondria and trichocysts. In previous
47
48
49 62 studies, monoclonal antibodies against *P. bursaria* trichocysts and mitochondria were
50
51 63 obtained; both mitochondrial and trichocyst densities of algae-bearing *P. bursaria* were
52
53
54 64 significantly lower than those of algae-free cells (Kodama and Fujishima 2011, 2022).
55
56 65 These results indicate that symbiotic algae compete for their attachment sites with
57
58
59 66 preexisting trichocysts and mitochondria, and algae have the ability to ensure algal
60

1
2
3
4
5
6 67 attachment sites beneath the host cell cortex (Kodama and Fujishima 2011).
7
8
9 68 Furthermore, high-speed centrifugation can induce rapid algal detachment from the host
10
11 69 cell cortex and concentrates the algae in the posterior end of the host cell (Kodama and
12
13 70 Fujishima 2013). Within 10 min of centrifugation, the detached algae recover their
14
15 71 original positions by host rapid cytoplasmic streaming. This algal reattachment was
16
17 72 inhibited when host cytoplasmic streaming was arrested by nocodazole. In nocodazole-
18
19 73 treated cells, approximately 5 h was required for complete algal recovery, and the host
20
21 74 cytoplasmic streaming had been resumed at that time. These results demonstrated that
22
23 75 adhesion of the PV beneath the host cell cortex can be repeatedly induced and that host
24
25 76 cytoplasmic streaming facilitates the recovery of algal attachment. However, the
26
27 77 mechanism by which the PV attaches beneath the host cell cortex remains unknown. In
28
29 78 this study, to investigate the mechanisms of symbiotic algal adhesion, the distribution of
30
31 79 mitochondria and trichocysts during reattachment was examined by
32
33 80 immunofluorescence microscopy using monoclonal antibodies against the mitochondria
34
35 81 and trichocysts. Furthermore, mitochondria of the anterior part of the host without
36
37 82 symbiotic algae were also observed.
38
39
40
41
42
43
44
45
46
47
48

84 **Materials and methods**

85 **Organism cultivation**

86 *Paramecium bursaria* strain Yad1g1N cells (syngen B1 or R3, mating type I) harboring
87 the symbiotic *Chlorella variabilis* strain 1N were used (Kodama and Fujishima 2009,
88 2011). *Paramecium* cells were cultivated in red pea (*Pisum sativum*) extract culture

1
2
3
4
5
6 89 medium (Tsukii *et al.* 1995) with a modified Dryl's solution (Dryl 1959; ~~(KH₂PO₄~~
7
8 90 was used instead of NaH₂PO₄·2H₂O) and inoculated with *Klebsiella aerogenes* (ATCC
9
10
11 91 35028) one day before use. The cultures were in the early stationary phase of growth
12
13 92 one day after the final feeding. All cells used in this study were in this phase.
14
15
16 93 Cultivation was performed in test tubes (18 mm × 180 mm) at 25 ± 1°C under
17
18 94 fluorescent lighting at 20–30 μmol photons m⁻²s⁻¹ using an incandescent lamp.
19
20

21 95

22 23 96 **Transmission electron microscopy (TEM)**

24
25
26 97 Algae-bearing *P. bursaria* were pre-fixed with 2% glutaraldehyde and prepared for
27
28 98 TEM, as described previously (Kodama *et al.* 2011). The paramecia embedded in
29
30 99 Spurr's resin (1969) were sectioned (70 nm thickness) using an ultramicrotome
31
32
33
34 100 (Reichert Ultracut S; Leica Microsystems, Vienna, Austria) with a diamond knife,
35
36 101 mounted on nickel mesh grids, and stained with lead citrate (Reynolds 1963). The
37
38 102 sections were observed using TEM (CM120; Philips) at 80 kV.
39
40

41 103

42 43 104 **High-speed centrifugation of *P. bursaria* cells**

44
45
46 105 Algae-bearing *P. bursaria* cells were centrifuged at high speed (Kodama and Fujishima
47
48 106 2011). *P. bursaria* cells at a density of 5 × 10³ cells/mL were placed in a 1.5 mL
49
50
51 107 microcentrifuge tube. The tube was then centrifuged using a fixed-angle rotor at 1000 ×
52
53 108 g for 1 min at 25 ± 1° C (Model 3740; Kubota Corporation, Tokyo, Japan). Before,
54
55 109 immediately after (approx. 3 min), 10 min, and 30 min after the centrifugation, aliquots
56
57
58 110 of *Paramecium* cells were air-dried on cover glasses (4.5 mm × 24 mm) and fixed with
59
60

1
2
3
4
5
6 111 4% (w/v) paraformaldehyde dissolved in phosphate-buffered saline (PBS) (137 mM
7
8 112 NaCl, 2.68 mM KCl, 8.1 mM NaHPO₄·12H₂O, 1.47 mM KH₂PO₄, pH 7.2) for 10 min
9
10
11 113 at 4°C.
12

13
14 114

15 16 115 **Indirect immunofluorescence microscopy**

17
18 116 The cover glasses with the fixed *P. bursaria* cells were washed with PBS containing
19
20
21 117 0.05% (v/v) Tween 20 (PBST) for 10 min at 4°C. The cells on the cover glasses were
22
23 118 treated with a culture supernatant of hybridoma cells containing either monoclonal
24
25
26 119 antibodies mAb5A11E2 against trichocysts of *P. bursaria* (Kodama and Fujishima
27
28 120 2011) or mAb2B8A8H1 against mitochondria of *P. bursaria* (Kodama and Fujishima
29
30
31 121 2022) overnight at 4°C, washed with PBS, and treated with Alexa Fluor 488 conjugated
32
33 122 goat anti-mouse IgG (Molecular Probes Inc., Eugene, OR, USA) diluted 1000-fold with
34
35
36 123 PBS for 2 h at 25°C. The cover glasses were then washed with PBS and observed under
37
38 124 a differential interference contrast microscope and fluorescence microscope (BX53;
39
40
41 125 Olympus Corp., Tokyo, Japan). Images were acquired using an Olympus DP74 system
42
43
44 126 and analyzed using Olympus cellSens Dimension software.
45

46 127

47 48 49 128 **Statistical analysis**

50
51 129 Data were analyzed using the Mann-Whitney U-test in R. Reproducibility of the data
52
53 130 was confirmed by three independent experiments.
54

55
56 131

57 58 132 **Results**

1
2
3
4
5
6 **133 Transmission electron microscopy (TEM) of algae-bearing *P. bursaria***

7
8
9 **134** Algae-bearing paramecia were observed using TEM (Fig. 1). As shown by the
10
11 **135** arrowhead in Fig. 1, we observed mitochondria that attach to the PVs, as shown in
12
13 **136** previous studies (Fujishima and Kodama 2012; Song *et al.* 2017). In contrast, the
14
15
16 **137** trichosyst membrane did not attach to the PV membrane (black arrow in Fig. 1).
17
18
19 **138**

20
21 **139 Effects of high-speed centrifugation on distribution of trichocysts in**
22
23 **140 algae-bearing *P. bursaria* cells**

24
25
26 **141** Figure 2A shows the results obtained by indirect immunofluorescence microscopy after
27
28 **142** high-speed centrifugation of algae-bearing *P. bursaria* cells using monoclonal
29
30 **143** antibodies against trichocysts. Before centrifugation, symbiotic algae were shown
31
32
33 **144** distributed throughout the host cell by differential interference contrast microscopy, and
34
35
36 **145** immunofluorescence showed that the trichocysts of the algae-bearing cells were
37
38 **146** localized as a ring surrounding the algae, as shown in a previous study (Kodama and
39
40 **147** Fujishima 2011). Immediately after centrifugation, the symbiotic algae dislocated and
41
42
43 **148** accumulated in the posterior region of *Paramecium*. As for trichocysts,
44
45
46 **149** immunofluorescence was hardly observed in the centrifuged cells. When cells are
47
48
49 **150** stimulated, trichocysts discharge their contents in milliseconds (Adoutte 1988).
50
51
52 **151** Therefore, most trichocysts are discharged via high-speed centrifugation. In fact, we
53
54 **152** observed many discharged trichocysts around the *Paramecium* cells (Fig. 2B). The
55
56
57 **153** monoclonal antibodies also distinguished after discharge, coinciding with findings in a
58
59
60

1
2
3
4
5
6 154 previous study (Kodama and Fujishima 2011) (Fig. 2B, IM). Ten minutes after
7
8 155 centrifugation, the algae completely localized beneath the host cortex, and few
9
10
11 156 trichocysts were observed by immunofluorescence microscopy. Thirty minutes after
12
13 157 centrifugation, trichocysts were barely observed. In fact, it was previously reported that
14
15 158 when the trichocysts are removed by treatment with lysozyme, regeneration of the
16
17 159 mature trichocysts begins at 3 h, even in the presence of lysozyme (Kodama and
18
19
20
21 160 Fujishima 2009). Therefore, it seems that 30 min are not sufficient for the recovery of
22
23 161 trichocysts. These results clearly support previous studies (Kodama and Fujishima
24
25 162 2009) that the symbiotic algae do not need trichocysts to attach beneath the host cell
26
27 163 cortex.

30
31 164 Figure 2C shows the immunofluorescence intensity of *P. bursaria* cells before (x-
32
33 165 bar, T-B), immediately after (x-bar, T-Ia), 10 min (x-bar, T-10 min), and 30 min (x-bar,
34
35 166 T-30 min) after centrifugation. The immunofluorescence intensity of the centrifuged
36
37 167 cells decreased drastically and did not increase until 30 min after centrifugation. These
38
39 168 quantitative data correspond well with the images shown in Fig. 2A. These quantitative
40
41 169 data correspond well with the results of indirect immunofluorescence microscopy, as
42
43 170 shown in Fig. 2A.

44
45
46
47
48
49 171

50
51 172

52
53 173 **Effects of high-speed centrifugation on distribution of mitochondria in**
54
55
56 174 **algae-bearing *P. bursaria* cells**

1
2
3
4
5
6 175 We examined the effect of high-speed centrifugation on host mitochondria by
7
8
9 176 immunofluorescence microscopy using the same method described above (Fig. 3).
10
11 177 Before centrifugation, immunofluorescence of mitochondria was observed around the
12
13 178 symbiotic algae. Immediately after centrifugation (approximately 3 min), the symbiotic
14
15 179 algae were dislocated, as shown above. Immunofluorescence was observed throughout
16
17
18 180 the cytoplasm. Note that the cortical mitochondrial immunofluorescence became strong
19
20
21 181 after centrifugation. This phenomenon may be due to better accessibility for
22
23
24 182 visualization of the labeling after detachment of the PV from the cortex. Ten and 30 min
25
26 183 after centrifugation, symbiotic algae were distributed throughout the cell because of
27
28 184 algal relocation. Immunofluorescence of mitochondria was also observed around the
29
30 185 symbiotic algae. These observations suggest that symbiotic algae do not need
31
32
33 186 trichocysts for their attachment but are colocalized with mitochondria ~~for their~~
34
35
36 187 ~~attachment~~, as shown by TEM observations (Fig. 1).
37
38

39 188 Figure 3B shows the immunofluorescence intensity of algae-free *P. bursaria* cells
40
41 189 before (x-bar, M-B), immediately after (x-bar, M-Ia), 10 min (x-bar, M-10 min), and 30
42
43 190 min (x-bar, M-30 min) after centrifugation. There was almost no change in
44
45
46 191 immunofluorescence intensity, and no significant difference was observed between the
47
48
49 192 data for each time point. These quantitative data correspond well with the images shown
50
51 193 in Fig. 3A ~~This quantitative data corresponds well with the results of indirect-~~
52
53
54 194 ~~immunofluorescence microscopy, as shown in Fig. 3A.~~
55

56 195

57
58 196 **Mitochondrial distribution of host anterior cortex**
59
60

1
2
3
4
5
6 197 Kodama (2013) investigated the symbiotic algal distribution of 14 strains of *P.*
7
8 198 *bursaria*. As a result, all strains had symbiotic algae at the ventral, dorsal, and posterior
9
10
11 199 cortexexies, and some cells did not have symbiotic algae at the anterior cortex. This
12
13
14 200 phenomenon is not strain-, syngen-, or mating-type specific. Only 35% of the strain
15
16 201 *Yad1g1N* cells, also used in this experiment, had symbiotic algae at their anterior
17
18 202 cortex. This paper also reported that the artificial trichocyst-discharge experiments by
19
20
21 203 the treatment of lysozyme clarified that trichocysts of the anterior cortex are difficult to
22
23
24 204 remove. Although the high-speed centrifugation experiment clearly showed that
25
26 205 trichocysts are unnecessary for algal reattachment, trichocyst-discharge difficulty of the
27
28 206 anterior cortex may be related to the restrict enrichment of the large PVs. It is unclear
29
30
31 207 why algae have difficulty adhering to the anterior cortex of their hosts. The high-speed
32
33
34 208 centrifugation experiment clearly showed that trichocysts are unnecessary for algal
35
36 209 reattachment.

37
38 210 On the other hand, host mitochondria remained unchanged in localization by high-
39
40
41 211 speed centrifugation. This indicated that the presence of mitochondria might may be
42
43
44 212 essential-related for algal adhesion. To determine whether the reason for the algal
45
46 213 difficulty in adhering to the host anterior cortex is due to the low mitochondrial density
47
48 214 in this area, we examined whether there were differences in the number and distribution
49
50
51 215 of mitochondria between cells with and without symbiotic algae in their anterior part
52
53
54 216 using indirect immunofluorescence microscopy with the monoclonal antibodies against
55
56 217 host mitochondria.
57
58
59
60

1
2
3
4
5
6 218 Figure 4 shows differential interference contrast microscopy images of *P. bursaria*
7
8 219 cells with (Algae+) and without (Algae-) symbiotic algae in the anterior end and
9
10 220 immunofluorescence images of those cells using mitochondrial monoclonal antibodies.
11
12
13 221 Note that mitochondria are present in the anterior end without symbiotic algae as well
14
15
16 222 as in the anterior end with symbiotic algae. This observation shows that not all areas
17
18 223 with mitochondria had algae, but there was a localization bias within the host cell.
19
20

21 224

22 225 **Discussion**

23
24
25
26 226 Localization of endosymbionts near the host cell cortex is a universal phenomenon; the
27
28 227 same phenomenon has been observed in other ciliate -algae or -cyanobacteria
29
30 228 endosymbionts, such as *Mayorella viridis*, *Coleps hirtus*, *Coleps spetai*, *Frontonia*
31
32 229 *leucas*, *Malacophrys sphagni*, *Ophrydium versatile*, *Vorticella* sp., *Climacostomum*
33
34 230 *virens*, *Euplotes daidaleos*, *Halteria bifurcata*, *Stentor polymorphus*, and *Stentor niger*
35
36 231 (Reisser 1986). Furthermore, the ability to adjust the intracellular symbiont position is
37
38 232 likely an important means of optimizing carbon production (Petrou *et al.* 2017).
39
40

41 233 A previous study showed that the infectivity of *Chlorella* species in *P. bursaria* is
42
43 234 based on their ability to localize beneath the host cell membrane after escaping from the
44
45 235 host digestive vacuole during the early infection process (Kodama and Fujishima 2007).
46
47 236 Furthermore, this algal attachment may be related to the avoidance of host lysosomal
48
49 237 digestion or fusion because algal digestion when the symbiotic algae are attached
50
51 238 beneath the host cell cortex has not been observed (Kodama and Fujishima unpubl.
52
53 239 data).
54
55
56
57
58
59
60

1
2
3
4
5
6 240 Acidosomes are organelles responsible for the acidification of DVs before
7
8
9 241 lysosomal fusion and are distributed throughout the cell (Allen 1993; Kodama 2013).
10
11 242 High-speed centrifugation can also induce the rapid accumulation of host acidosomes
12
13 243 and lysosomes at the anterior end of *Paramecium*. Within 10 min of centrifugation, the
14
15 244 accumulated vesicles recover their original positions by host rapid cytoplasmic
16
17 245 streaming (Kodama 2013). This observation showed that although acidosomes and
18
19 246 lysosomes were dislocated by centrifugation, the mitochondria remained unchanged.
20
21 247 This is probably because acidosomes and lysosomes undergo cytoplasmic streaming,
22
23 248 but mitochondria do not.

24 249 Figure 5 shows a schematic representation of the algal attachment mechanism
25
26 250 beneath the host cell cortex after dislocation via high-speed centrifugation. After the
27
28 251 high-speed centrifugation, almost all trichocysts are released out of the cell. The
29
30 252 discharged trichocysts were regenerate from the endoplasmic reticulum, pass through
31
32 253 the Golgi apparatus, and undergo a maturation process (pretrichocysts) before mature
33
34 254 trichocysts are delivered to the cell membrane (Plattner 2017). Pretrichocysts contain a
35
36 255 growing mass of electron dense secretory material. The pretrichocysts then elongate
37
38 256 while their luminal space is progressively filled with crystallizing secretory materials
39
40 257 (Garreau De Loubresse 1993). Because several hours are required for regeneration after
41
42 258 trichocyst discharge (Harumoto 2002), no trichocysts are observed in the cells after
43
44 259 algal reattachment. This representation can be defined by obtaining monoclonal
45
46 260 antibodies against both mitochondria and trichocysts. To the best of our knowledge, this
47
48
49
50
51
52
53
54
55
56
57
58
59
60

1
2
3
4
5
6 261 is the first report showing the distribution of host mitochondria and trichocysts during
7
8 262 algal relocation beneath the host cell cortex.

9
10
11 263 Fast cytoplasmic streaming after high-speed centrifugation of *P. bursaria* is the
12
13 264 driving force for algal relocation. The characteristic feature of rotational cytoplasmic
14
15 265 streaming in the genus *Paramecium* (*P. aurelia*, *P. caudatum*, *P. bursaria*, *P.*
16
17 266 *multimicronucleatum*, and *P. calkinsi*) is that the pattern of its route and direction
18
19 267 remain constant (Sikora 1981). Various factors, such as cell division, conjugation,
20
21 268 temperature, and osmotic pressure, are known to affect cytoplasmic streaming in
22
23 269 *Paramecium* (Sikora 1981), but the detachment of the symbiotic algae by high-speed
24
25 270 centrifugation is also a factor. In *P. bursaria*–*Chlorella* endosymbiosis, their cell cycle
26
27 271 pace is guaranteed to be synchronous (Takahashi 2016). Takahashi *et al.* (2007) found
28
29 272 that *P. bursaria* controls the proliferation of endosymbiotic algae through host-cell
30
31 273 cycle-dependent cytoplasmic streaming. Thus, host cytoplasmic streaming plays a major
32
33 274 role in maintaining the number and cellular localization of symbiotic algae in *P.*
34
35 275 *bursaria* cells.

36
37
38 276 The mitochondria of the host *P. bursaria* are close to the PV membrane (Fig. 1),
39
40 277 and their number and function are altered by the presence or absence of symbiotic algae
41
42 278 (Kodama and Fujishima 2022). Therefore, host mitochondria and symbiotic algae are
43
44 279 expected to be strongly associated with each other. We assumed that the presence of
45
46 280 mitochondria was essential for the existence of symbiotic algae; however, the results in
47
48 281 Fig. 4 contradict this expectation. Mitochondria and endoplasmic reticulum (ER) are
49
50 282 contact at ER-Mitochondria Contact Sites (EMCS)s. Lipids and Ca²⁺ synthesized in the
51
52
53
54
55
56
57
58
59
60

1
2
3
4
5
6 283 ER are transported to mitochondria through the EMCSs (Kornmann *et al.* 2009; Rizzuto
7
8 284 *et al.* 1998). Furthermore, EMCs is also important in reactive oxygen species (ROS)
9
10 285 production (Booth *et al.* 2016) and autophagosome formation (Hamasaki *et al.* 2013).
11
12 286 Song *et al.* (2017) reported that the host ~~endoplasmic reticulum~~ER is also involved in
13
14 287 organizing intracellular algal symbiosis in the cytoplasm. The relationship between host
15
16 288 mitochondria and symbiotic algae and the relationship between other cellular
17
18 289 organelles, including the host endoplasmic reticulum, should be examined in detail in
19
20 290 future studies.

21
22
23
24
25
26 291 The exposure of *P. bursaria* cells to ultraviolet and photosynthetically active
27
28 292 radiation is known to induce symbiotic algal dislocation, moving to the posterior cell
29
30 293 region (Summerer *et al.* 2009). The displaced algae relocate to a "normal" distribution
31
32 294 when host cells are transferred to a medium without ultraviolet radiation conditions. The
33
34 295 mechanisms of algal displacement and relocation remain unclear (Summerer *et al.*
35
36 296 2009). A dislocation of *Chlorella* symbionts has also been described in the ciliate
37
38 297 *Pelagodileptus trachelioides* (Butkay 2004). Although this phenomenon appears to be a
39
40 298 stress reaction followed by host cytolysis, the details remain unknown. The method of
41
42 299 artificial algal detachment and subsequent rapid reattachment by high-speed
43
44 300 centrifugation of algae-bearing *P. bursaria* may contribute to understanding the
45
46 301 mechanism of symbiotic algal localization in a variety of host species.

47
48 302 Although the mechanism of PV localization beneath the host cell cortex is still
49
50 303 unknown, the docking or distribution mechanism of mitochondria and uninserted
51
52 304 trichocysts in other ciliate species, such as *Paramecium tetraurelia*, have been reported.

1
2
3
4
5
6 305 Aufderheide (1977) reported that, in the subcortical regions of *P. tetraurelia*,
7
8 306 mitochondria and uninserted (undocked) trichocysts display saltatory motility with
9
10 307 individual characteristics, making them distinguishable from each other and from
11
12 308 cellular cyclosis. The saltatory motion of trichocysts is implicated as a means of
13
14 309 transporting new trichocysts from the cytoplasm to their ultimate location in the cellular
15
16 310 cortex. In addition, the saltatory motion may be a factor in the intracellular distribution
17
18 311 of the mitochondria. The mechanism of trichocyst docking at the *Paramecium* cell
19
20 312 membrane has been reported in detail (Plattner 2017; Plattner *et al.* 1982). Before
21
22 313 trichocyst docking, mature trichocysts move through the subcortical region by cyclosis
23
24 314 (Sikora 1981). Then, they approach, in a saltatory manner, tip first, a docking site at the
25
26 315 cell membrane (Aufderheide 1978); Thereby, trichocysts are guided by the same
27
28 316 “hand.” This entity is a microtubule emanating from a nearby ciliary basal body
29
30 317 (Plattner *et al.* 1982), which always is situated ~ 0.5 to 1 μm from a docking site proper.
31
32 318 Petrou *et al.* (2017) found that the intracellular algal symbionts within the large
33
34 319 photosymbiotic foraminifera *Marginopora vertebralis* exhibit phototactic behavior and
35
36 320 the phototactic movement of the symbionts is accomplished by the host through rapid
37
38 321 actin-mediated relocation of the symbionts deeper into the cavities within the calcite
39
40 322 skeletons. Thus, the host cytoskeleton plays a major role in the migration of host
41
42 323 cytosolic organelles and symbiotic algae. In order to study the involvement of the
43
44 324 cytoskeleton in the process of PV localization, the cell cortex of algae-bearing *P.*
45
46 325 *bursaria* cells was observed in detail by TEM. However, no cytoskeletal structure that
47
48 326 seems to be related to PV localization has been found until now (Kodama unpub. Data).
49
50
51
52
53
54
55
56
57
58
59
60

1
2
3
4
5 327 Adhesion just below the host cell surface is a universal phenomenon found not
6
7 328 only in cell organelles and symbiotic algae but also in pathogenic bacteria. Protists are
8
9 329 potential natural hosts of various bacterial species, including pathogens. *Paramecium*
10
11 330 *caudatum* could be a natural reservoir of *Legionella pneumophila* (Watanabe *et al.*
12
13 331 2016), and *P. bursaria* could be a potential host of *Francisella novicida* (Watanabe *et*
14
15 332 *al.* 2022). *Francisella novicida* is a facultative intracellular pathogen and a causative
16
17 333 agent of tularemia (Ellis *et al.* 2002). Recently, Watanabe *et al.* (2022) found that *F.*
18
19 334 *novicida* (strain U112) cells, which were wrapped with the host DV membrane,
20
21 335 localized beneath the host cell cortex. This indicates that both symbiotic algae and
22
23 336 bacteria can attach beneath the host cell cortex. Therefore, elucidation of the mechanism
24
25 337 of localization beneath the host cell cortex using *P. bursaria* is expected to contribute to
26
27 338 the development of not only symbiotic biology but also infectious disease research.
28
29
30
31
32

33
3435
36 **References**37
38 341 Adoutte A. Exocytosis: Biogenesis, Transport and Secretion of Trichocysts. In: Görtz
39
40 342 HD (ed). *Paramecium*. Springer: Berlin, Heidelberg, 1988, 325–62.
4142
43 34344
45 344 Allen RD. Acidosomes: recipients of multiple sources of membrane and cargo during
46
47 345 development and maturation. *J Cell Sci* 1993;**106**:411–22.
4849
50 34651
52 347 Alberts D, Wiessner W. Nitrogen nutrition of endosymbiotic *Chlorella spec.* *Endocyt*
53
54 348 *Cell Res* 1985;**1**:55–64.
5556
57 349
58
59
60

- 1
2
3
4
5 350 Albers D, Reisser W, Wiessner W. Studies on the nitrogen supply of endosymbiotic
6
7 351 chlorellae in green *Paramecium bursaria*. *Plant Sci Lett* 1982;**25**:85–90.
8
9
10 352
11
12 353 Aufderheide KJ. Saltatory motility of uninserted trichocysts and mitochondria in
13
14 354 *Paramecium tetraurelia*. *Science* 1977;**198**:299–300.
15
16
17 355
18
19 356 Aufderheide KJ. Motility events of trichocyst insertion in *Paramecium tetraurelia*. *J*
20
21 357 *Protozool* 1978;**25**:362–65.
22
23
24 358
25
26 359 Blanc G, Duncan G, Agarkova I *et al.* The *Chlorella variabilis* NC64A genome reveals
27
28 360 adaptation to photosynthesis, coevolution with viruses, and cryptic sex. *The Plant Cell*
29
30 361 2010;**22**:2943–55.
31
32
33 362
34
35 363
36
37 364 Booth DM, Enyedi B, Geiszt M *et al.* Redox Nanodomains Are Induced by and Control
38
39 365 Calcium Signaling at the ER-Mitochondrial Interface. *Mol Cell* 2016;**63**:240–48.
40
41
42 366
43
44 367 Brown JA, Nielsen PJ. Transfer of photosynthetically produced carbohydrate from
45
46 368 endosymbiotic *Chlorellae* to *Paramecium bursaria*. *J Protozool* 1974;**21**:569–70.
47
48
49 369
50
51 370 Butkay M. Beobachtungen an *Pelagodileptus trachelioides* (Ciliophora). *Lauterbornia*
52
53 371 2004;**49**:129–40.
54
55
56 372
57
58
59
60

1
2
3
4
5 373 Cheng YH, Liu CFJ, Yu YH *et al.* Genome plasticity in *Paramecium bursaria* revealed
6
7 374 by population genomics. *BMC Biology* 2020;**18**:180.
8
9

10 375
11
12 376 Dryl S. Antigensic transformation in *Paramecium aurelia* after homologous antiserum
13
14 377 treatment during autogamy and conjugation. *J Protozool* 1959;**6**:25.
15
16

17 378
18
19 379 Ellis J, Oyston PC, Green M *et al.* Tularemia. *Clin Microbiol Rev* 2002;**15**:631–46.
20
21

22 380
23
24 381 Fujishima M, Kodama Y. Endosymbionts in *Paramecium*. *Europ J Protistol*
25
26 382 2012;**48**:124–37.
27
28

29 383
30 384 Garreau De Loubresse N. Early steps of the secretory pathway in *Paramecium*:
31
32 ultrastructural, immunocytochemical, and genetic analysis of trichocyst biogenesis. In:
33 385 Plattner H (ed). *Membrane Traffic in Protozoa*, JAI Press, Greenwich (CT, USA),
34
35 386 London (GB), 1993, 27–59.
36
37 387
38
39

40 388
41
42 389
43
44 390 Gortz HD. Infections of *Paramecium bursaria* with bacteria and yeasts. *J Cell Sci* 1982;
45
46 391 **58**:445–53.
47
48

49 392
50
51 393 Gu FK, Chen L, Ni B, Zhang X. A comparative study on the electron microscopic
52
53 394 enzymo-cytochemistry of *Paramecium bursaria* from light and dark cultures. *Eur J*
54
55 395 *Protistol* 2002;**38**:267–78.
56
57

58 396
59
60

397

398 [Hamasaki M, Furuta N, Matsuda A et al. Autophagosomes form at ER-mitochondria](#)

399 [contact sites. *Nature* 2013;**495**:389–93.](#)

400

401 [Harumoto T. Defensive function of trichocysts in *Paramecium*. *Jpn J Protozool*](#)

402 [2002;**35**:125–33.](#)

403

404 Karakashian SJ, Rudzinska MA. Inhibition of lysosomal fusion with symbiont-

405 containing vacuoles in *Paramecium bursaria*. *Exp Cell Res* 1981;**131**:387–93.

406

407 Kodama Y. Localization of attachment area of the symbiotic *Chlorella variabilis* of the

408 ciliate *Paramecium bursaria* during the algal removal and reinfection. *Symbiosis*

409 2013;**60**:25–36.

410 Kodama Y, Fujishima M. Infectivity of *Chlorella* species for the ciliate *Paramecium*

411 *bursaria* is not based on sugar residues of their cell wall components, but on their

412 ability to localize beneath the host cell membrane after escaping from the host digestive

413 vacuole in the early infection process. *Protoplasma* 2007;**231**:55–63.

414

415 Kodama Y, Fujishima M. Localization of perialgal vacuoles beneath the host cell

416 surface is not a prerequisite phenomenon for protection from the host's lysosomal fusion

417 in the ciliate *Paramecium bursaria*. *Protist* 2009;**160**:319–29.

418

419 Kodama Y, Fujishima M. Secondary symbiosis between *Paramecium* and *Chlorella*

420 cells. *Int Rev Cell Mol Biol* 2010;**279**:33–77.

1
2
3
4
5 421
6

7 422 Kodama Y, Fujishima M. Endosymbiosis of *Chlorella* species to the ciliate

8 423 *Paramecium bursaria* alters the distribution of the host's trichocysts beneath the host
9
10
11
12 424 cell cortex. *Protoplasma* 2011;**248**:325–37.
13

14 425

15
16 426 Kodama Y, Fujishima M. Synchronous induction of detachment and reattachment of

17
18 427 symbiotic *Chlorella* spp. from the cell cortex of the host *Paramecium bursaria*. *Protist*
19
20 428 2013;**164**:660–72.
21
22

23 429

24
25 430 Kodama Y, Fujishima M. Endosymbiotic *Chlorella variabilis* reduces mitochondrial

26
27 431 number in the ciliate *Paramecium bursaria*. *Sci Rep* 2022;**12**:8216.
28
29

30 432

31
32 433 Kodama Y, Inouye I, Fujishima M. Symbiotic *Chlorella vulgaris* of the ciliate

33
34 434 *Paramecium bursaria* plays an important role in maintaining perialgal vacuole
35
36 435 membrane functions. *Protist* 2011;**162**:288–303.
37
38

39 436

40
41
42 437 [Kornmann B, Currie E, Collins SR et al. An ER-mitochondria tethering complex](#)

43
44 438 [revealed by a synthetic biology screen. *Science* 2009;**325**:477–81.](#)
45
46

47 439

48
49 440 Petrou K, Ralph PJ, Nielsen DA. A novel mechanism for host-mediated photoprotection

50
51 441 in endosymbiotic foraminifera. *ISME J* 2017;**11**:453–62.
52
53

54 442
55
56
57
58
59
60

- 1
2
3
4
5 443 Plattner H. Trichocysts-*Paramecium*'s Projectile-like Secretary Organelles: Reappraisal
6
7 444 of their Biogenesis, Composition, Intracellular Transport, and Possible Functions. *J*
8
9 445 *Eukaryot Microbiol* 2017;**64**:106–33.
10
11
12 446
13
14 447 Plattner H, Westphal C, Tiggemann R. Cytoskeleton secretary vesicle interactions
15
16 448 during the docking of secretary vesicles at the cell membrane in *Paramecium*
17
18 449 *tetraurelia* cells. *J Cell Biol* 1982;**92**:368–77.
19
20
21 450
22
23 451 Reisser W. Endosymbiotic associations of freshwater protozoa and algae. In: Corliss
24
25 452 JO, Patterson DJ (eds). *Progress in Protistology*, Biopress Ltd, Bristol, 1986, 195–214.
26
27
28 453
29
30 454
31
32 455 Rizzuto R, Pinton P, Carrington W et al. Close contacts with the endoplasmic reticulum
33
34 456 as determinants of mitochondrial Ca²⁺ responses. *Science* 1998;**280**:1763–66.
35
36
37 457
38
39 458 Sikora J. Cytoplasmic streaming in *Paramecium*. *Protoplasma* 1981;**109**:57–77.
40
41
42 459
43
44 460 Song C, Murata K, Suzaki T. Intracellular symbiosis of algae with possible involvement
45
46 461 of mitochondrial dynamics. *Sci Rep* 2017;**7**:1221.
47
48
49 462
50
51 463
52
53 464 Summerer M, Sonntag B, Hörtnagl P et al. Symbiotic ciliates receive protection against
54
55 465 UV damage from their algae: A test with *Paramecium bursaria* and *Chlorella*. *Protist*
56
57 466 2009;**160**:233–43.
58
59
60

- 1
2
3
4
5 467
6
7
8 468 Takahashi T. Simultaneous Evaluation of Life Cycle Dynamics between a Host
9
10 469 *Paramecium* and the Endosymbionts of *Paramecium bursaria* Using Capillary Flow
11
12 470 Cytometry. *Sci Rep* 2016;**6**:31638.
13
14 471
15
16 472 Takahashi T, Shirai Y, Kosaka T *et al.* Arrest of Cytoplasmic Streaming Induces Algal
17
18 473 Proliferation in Green *Paramecia*. *PLoS ONE* 2007;**12**:e1352.
19
20 474
21
22 475 Tsukii Y, Harumoto T, Yazaki K. Evidence for a viral macro- nuclear endosymbiont in
23
24 476 *Paramecium caudatum*. *J Euk Microbiol* 1995;**42**:109–15.
25
26 477
27
28 478 Reisser W. The metabolic interactions between *Paramecium bursaria* Ehrbg. and
29
30 479 *Chlorella* spec. in the *Paramecium bursaria*-symbiosis. II. Symbiosis-specific
31
32 480 properties of the physiology and the cytology of the symbiotic unit and their regulation
33
34 481 (author's transl). *Arch Microbiol* 1976;**111**:161–70.
35
36 482
37
38 483 Reisser W. The metabolic interactions between *Paramecium bursaria* Ehrbg. and
39
40 484 *Chlorella* spec. in the *Paramecium bursaria*-symbiosis. III. The influence of different
41
42 485 CO₂-concentrations and of glucose on the photosynthetic and respiratory of the
43
44 486 symbiotic unit. *Arch Microbiol* 1980;**125**:291–93.
45
46 487
47
48 488 Reisser W. Endosymbiotic associations of freshwater protozoa and algae. *Prog Protistol*
49
50 489 1986;**1**:195–214.
51
52 490
53
54
55
56
57
58
59
60

- 1
2
3
4
5 491 Reynolds ES. The use of lead citrate at high pH as an electron-opaque stain in electron
6
7 492 microscopy. *J Cell Biol* 1963;**17**:208–12.
8
9
10 493
11
12 494 Spurr AR. A low viscosity epoxy resin embedding medium for electron microscopy. *J*
13
14 495 *Ultrastruct Res* 1969;**26**:31–43.
15
16
17 496
18
19 497
20
21 498 Watanabe K, Nakao R, Fujishima M *et al.* Ciliate *Paramecium* is a natural reservoir of
22
23 499 *Legionella pneumophila*. *Sci Rep* 2016;**6**:24322.
24
25
26 500
27
28 501 Watanabe K, Motonaga A, Tachibana M *et al.* *Francisella novicida* can
29
30 502 utilize *Paramecium bursaria* as its potential host. *Envl Microbiol Rep* 2022;**14**:50–9.
31
32
33
34
35
36
37
38
39
40
41
42
43
44
45
46
47
48
49
50
51
52
53
54
55
56
57
58
59
60

1
2
3
4
5
6 1 **Role of host ciliate *Paramecium bursaria* mitochondria and trichocysts for**
7
8 **2 symbiotic *Chlorella variabilis* attachment beneath the host cell cortex**
9
10
11 3
12
13
14 4 Mitochondria and trichocysts of the ciliate *Paramecium bursaria*
15
16 5
17
18
19 6 Yuuki Kodama^{1,*}, Masahiro Fujishima^{2,†,‡}

20
21 7 ¹Institute of Agricultural and Life Sciences, Academic Assembly, Shimane
22
23 8 University, 1060 Nishikawatsu-cho, Matsue-shi, Shimane 690-8504, Japan
24
25

26 9 ²Department of Environmental Science and Engineering, Graduate School of
27
28 10 Science and Engineering, Yamaguchi University, Yoshida 1677-1, Yamaguchi
29
30 11 753-8512, Japan
31
32

33 12 † Present address: Research Center for Thermotolerant Microbial Resources,
34
35 13 Yamaguchi University, Yoshida 1677-1, Yamaguchi 753-8515, Japan
36
37

38 14 ‡ Institute of Environmental Radioactivity, Fukushima University, Kanayagawa 1,
39
40 15 Fukushima 960-1296, Japan
41
42
43
44
45

46 17 **Corresponding author:** Yuuki Kodama, Institute of Agricultural and Life
47
48 18 Sciences, Academic Assembly, Shimane University, 1060 Nishikawatsu-cho,
49
50 19 Matsue-shi, Shimane 690-8504, Japan. Tel: -81-852-32-6438; E-mail:
51
52 20 kodama@life.shimane-u.ac.jp
53
54
55

56 21
57
58
59
60

1
2
3
4
5
6 22 **One sentence summary:** Although the host mitochondria of ciliate *Paramecium*
7
8 23 *bursaria* are near symbiotic algae during reattachment, this does not mean that the algae
9
10
11 24 can exist if mitochondria are present, indicating some localization bias within the host
12
13
14 25 cell.

15
16 2617
18 27 **Abstract**

19
20
21 28 Symbiotic *Chlorella variabilis* is encased in the perialgal vacuole (PV) membrane of
22
23
24 29 ciliate *Paramecium bursaria*. The PV membrane is stably anchored below the host cell
25
26 30 cortex by adhesion to host mitochondria. Host trichocysts, which are defensive
27
28 31 organelles against predators, are present in the mitochondria and PV membrane vicinity.
29
30
31 32 The mechanism by which PV attaches beneath the host cell cortex remains unknown.
32
33
34 33 When *P. bursaria* is centrifuged at high speed, the symbiotic algae are displaced from
35
36 34 the host cell cortex and concentrate at the posterior end. When centrifugation is stopped,
37
38 35 the dislocated algae reattach beneath the host cell cortex with fast cytoplasmic
39
40
41 36 streaming. The densities of mitochondria and trichocysts before and after centrifugation
42
43
44 37 were compared using indirect immunofluorescence microscopy with monoclonal
45
46 38 antibodies. Almost all trichocysts were shed by high-speed centrifugation, but
47
48 39 dislocated algae could reattach even in the absence of trichocysts. In contrast, host
49
50
51 40 mitochondria were unaffected in localization and number, and the dislocated algae also
52
53
54 41 reattached. These findings suggest trichocysts are unnecessary for algal re-localization_

55
56 42 and that mitochondria are colocalized with the algae, unlike mitochondria. However,
57
58
59 43 many mitochondria were also present in the cell's anterior region without symbiotic
60

1
2
3
4
5
6 44 algae. Therefore, not all areas with mitochondria contained algae, but there was a
7
8 45 localization bias within the host cell.
9

10
11 46

12
13 47 **Keywords:** Algal reattachment, *Chlorella variabilis*, Endosymbiosis, Mitochondria,
14
15
16 48 *Paramecium bursaria*, Trichocyst
17

18
19 49

20
21 50 **Introduction**
22

23 51 *Paramecium bursaria*, a freshwater ciliate, is a symbiotic organism that establishes
24
25 52 endosymbiotic relationships with *Chlorella* spp. Each symbiotic alga is enclosed in a
26
27 53 perialgal vacuole (PV) derived from the host digestive vacuole (DV), which protects the
28
29 54 alga from lysosomal fusion (Gu *et al.* 2002; Karakashian and Rudzinska 1981). This
30
31 55 relationship between ciliates and algae is mutualistic; the host cell supplies algae with
32
33 56 nitrogen and oxygen (Albers *et al.* 1982, 1985; Reisser 1976, 1980), whereas the algae
34
35 57 provide the host with photosynthetic products (Brown *et al.* 1974; Reisser 1986) and
36
37 58 CO₂ (Reisser 1980). However, both *P. bursaria* and symbiotic algae can live without
38
39 59 their partners. The re-establishment of endosymbiosis between algae-free (removed) *P.*
40
41 60 *bursaria* cells and symbiotic *Chlorella* cells isolated from algae-bearing hosts can be
42
43 61 induced (Kodama and Fujishima 2010). In addition to their original symbiotic algae,
44
45 62 algae-free *P. bursaria* cells can be reinfected with bacteria and yeast that are retained in
46
47 63 the cytoplasm (Görtz 1982; Watanabe *et al.* 2022). The nuclear genomes of the
48
49 64 symbiotic *Chlorella variabilis* (Blanc *et al.* 2010) and the host *P. bursaria* have been
50
51
52
53
54
55
56
57
58
59
60

1
2
3
4
5
6 65 explored (Cheng *et al.* 2020); these organisms are now considered models for
7
8 66 endosymbiosis research.

9
10
11 67 Using pulse-labeling and chasing of algae-free paramecia for 1.5 min with
12
13 68 symbiotic algae that were isolated from algae-bearing *P. bursaria*, four important
14
15 69 cytological events have been identified. These events are necessary for the
16
17 70 establishment of endosymbiosis and the timing of each mechanism during the infection
18
19 71 process (Kodama and Fujishima 2010). 1) Within 3 min of algal mixing, part of the
20
21 72 algae has resistance to the host's lysosomal digestive enzymes in the DVs. 2) Within 30
22
23 73 min of mixing, algae in the DV begin budding from the DV membrane into the
24
25 74 cytoplasm. 3) Within 15 min of budding from the DV, the DV membrane enclosing a
26
27 75 single green *Chlorella* differentiates into a PV membrane. 4) The PV membrane
28
29 76 translocates beneath the host cell cortex.

30
31
32
33
34
35
36 77 The PV appears to localize near the host mitochondria and trichocysts. In previous
37
38 78 studies, monoclonal antibodies against *P. bursaria* trichocysts and mitochondria were
39
40 79 obtained; both mitochondrial and trichocyst densities of algae-bearing *P. bursaria* were
41
42 80 significantly lower than those of algae-free cells (Kodama and Fujishima 2011, 2022).
43
44 81 These results indicate that symbiotic algae compete for their attachment sites with
45
46 82 preexisting trichocysts and mitochondria, and algae have the ability to ensure algal
47
48 83 attachment sites beneath the host cell cortex (Kodama and Fujishima 2011).
49
50 84 Furthermore, high-speed centrifugation can induce rapid algal detachment from the host
51
52 85 cell cortex and concentrates the algae in the posterior end of the host cell (Kodama and
53
54 86 Fujishima 2013). Within 10 min of centrifugation, the detached algae recover their
55
56
57
58
59
60

1
2
3
4
5
6 87 original positions by host rapid cytoplasmic streaming. This algal reattachment was
7
8 inhibited when host cytoplasmic streaming was arrested by nocodazole. In nocodazole-
9
10 treated cells, approximately 5 h was required for complete algal recovery, and the host
11
12 cytoplasmic streaming had been resumed at that time. These results demonstrated that
13
14
15
16 91 adhesion of the PV beneath the host cell cortex can be repeatedly induced and that host
17
18 92 cytoplasmic streaming facilitates the recovery of algal attachment. However, the
19
20 93 mechanism by which the PV attaches beneath the host cell cortex remains unknown. In
21
22 94 this study, to investigate the mechanisms of symbiotic algal adhesion, the distribution of
23
24 95 mitochondria and trichocysts during reattachment was examined by
25
26 96 immunofluorescence microscopy using monoclonal antibodies against the mitochondria
27
28 97 and trichocysts. Furthermore, mitochondria of the anterior part of the host without
29
30 98 symbiotic algae were also observed.
31
32
33
34
35

36 99

100 **Materials and methods**

101 **Organism cultivation**

102 *Paramecium bursaria* strain Yad1g1N cells (syngen B1 or R3, mating type I) harboring
103 the symbiotic *Chlorella variabilis* strain 1N were used (Kodama and Fujishima 2009,
104 2011). *Paramecium* cells were cultivated in red pea (*Pisum sativum*) extract culture
105 medium (Tsukii *et al.* 1995) with a modified Dryl's solution (Dryl 1959; ~~⋅~~)(KH_2PO_4
106 was used instead of $\text{NaH}_2\text{PO}_4 \cdot 2\text{H}_2\text{O}$) and inoculated with *Klebsiella aerogenes* (ATCC
107 35028) one day before use. The cultures were in the early stationary phase of growth
108 one day after the final feeding. All cells used in this study were in this phase.

1
2
3
4
5
6 109 Cultivation was performed in test tubes (18 mm × 180 mm) at 25 ± 1°C under

7
8 110 fluorescent lighting at 20–30 μmol photons m⁻²s⁻¹ using an incandescent lamp.

9
10
11 111

12 112 **Transmission electron microscopy (TEM)**

13
14
15
16 113 Algae-bearing *P. bursaria* were pre-fixed with 2% glutaraldehyde and prepared for

17
18 114 TEM, as described previously (Kodama *et al.* 2011). The paramecia embedded in

19
20 115 Spurr's resin (1969) were sectioned (70 nm thickness) using an ultramicrotome

21
22 116 (Reichert Ultracut S; Leica Microsystems, Vienna, Austria) with a diamond knife,

23
24 117 mounted on nickel mesh grids, and stained with lead citrate (Reynolds 1963). The

25
26 118 sections were observed using TEM (CM120; Philips) at 80 kV.

27
28
29
30
31 119

32 120 **High-speed centrifugation of *P. bursaria* cells**

33
34
35
36 121 Algae-bearing *P. bursaria* cells were centrifuged at high speed (Kodama and Fujishima

37
38 122 2011). *P. bursaria* cells at a density of 5 × 10³ cells/mL were placed in a 1.5 mL

39
40 123 microcentrifuge tube. The tube was then centrifuged using a fixed-angle rotor at 1000 ×

41
42 124 g for 1 min at 25 ± 1° C (Model 3740; Kubota Corporation, Tokyo, Japan). Before,

43
44 125 immediately after (approx. 3 min), 10 min, and 30 min after the centrifugation, aliquots

45
46 126 of *Paramecium* cells were air-dried on cover glasses (4.5 mm × 24 mm) and fixed with

47
48 127 4% (w/v) paraformaldehyde dissolved in phosphate-buffered saline (PBS) (137 mM

49
50 128 NaCl, 2.68 mM KCl, 8.1 mM NaHPO₄·12H₂O, 1.47 mM KH₂PO₄, pH 7.2) for 10 min

51
52 129 at 4°C.

53
54
55
56
57
58 130

131 **Indirect immunofluorescence microscopy**

132 The cover glasses with the fixed *P. bursaria* cells were washed with PBS containing
133 0.05% (v/v) Tween 20 (PBST) for 10 min at 4°C. The cells on the cover glasses were
134 treated with a culture supernatant of hybridoma cells containing either monoclonal
135 antibodies mAb5A11E2 against trichocysts of *P. bursaria* (Kodama and Fujishima
136 2011) or mAb2B8A8H1 against mitochondria of *P. bursaria* (Kodama and Fujishima
137 2022) overnight at 4°C, washed with PBS, and treated with Alexa Fluor 488 conjugated
138 goat anti-mouse IgG (Molecular Probes Inc., Eugene, OR, USA) diluted 1000-fold with
139 PBS for 2 h at 25°C. The cover glasses were then washed with PBS and observed under
140 a differential interference contrast microscope and fluorescence microscope (BX53;
141 Olympus Corp., Tokyo, Japan). Images were acquired using an Olympus DP74 system
142 and analyzed using Olympus cellSens Dimension software.

143

144 **Statistical analysis**

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

147

148 **Results**

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

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

1
2
3
4
5
6 152 previous studies (Fujishima and Kodama 2012; Song *et al.* 2017). In contrast, the
7
8 153 trichosyst membrane did not attach to the PV membrane (black arrow in Fig. 1).
9

10
11 154

12
13 155 **Effects of high-speed centrifugation on distribution of trichocysts in**
14
15
16 156 **algae-bearing *P. bursaria* cells**

17
18 157 Figure 2A shows the results obtained by indirect immunofluorescence microscopy after
19
20
21 158 high-speed centrifugation of algae-bearing *P. bursaria* cells using monoclonal
22
23 159 antibodies against trichocysts. Before centrifugation, symbiotic algae were shown
24
25
26 160 distributed throughout the host cell by differential interference contrast microscopy, and
27
28 161 immunofluorescence showed that the trichocysts of the algae-bearing cells were
29
30
31 162 localized as a ring surrounding the algae, as shown in a previous study (Kodama and
32
33 163 Fujishima 2011). Immediately after centrifugation, the symbiotic algae dislocated and
34
35
36 164 accumulated in the posterior region of *Paramecium*. As for trichocysts,
37
38
39 165 immunofluorescence was hardly observed in the centrifuged cells. When cells are
40
41
42 166 stimulated, trichocysts discharge their contents in milliseconds (Adoutte 1988).

43
44 167 Therefore, most trichocysts are discharged via high-speed centrifugation. In fact, we
45
46
47 168 observed many discharged trichocysts around the *Paramecium* cells (Fig. 2B). The
48
49 169 monoclonal antibodies also distinguished after discharge, coinciding with findings in a
50
51
52 170 previous study (Kodama and Fujishima 2011) (Fig. 2B, IM). Ten minutes after
53
54 171 centrifugation, the algae completely localized beneath the host cortex, and few
55
56
57 172 trichocysts were observed by immunofluorescence microscopy. Thirty minutes after
58
59
60

1
2
3
4
5
6 173 centrifugation, trichocysts were barely observed. In fact, it was previously reported that
7
8 174 when the trichocysts are removed by treatment with lysozyme, regeneration of the
9
10 175 mature trichocysts begins at 3 h, even in the presence of lysozyme (Kodama and
11
12 176 Fujishima 2009). Therefore, it seems that 30 min ~~are~~ not sufficient for the recovery of
13
14 177 trichocysts. These results clearly support previous studies (Kodama and Fujishima
15
16 178 2009) that the symbiotic algae do not need trichocysts to attach beneath the host cell
17
18 179 cortex.

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

187 188 189 **Effects of high-speed centrifugation on distribution of mitochondria in** 190 **algae-bearing *P. bursaria* cells**

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

1
2
3
4
5
6 195 algae were dislocated, as shown above. Immunofluorescence was observed throughout
7
8 196 the cytoplasm. Note that the cortical mitochondrial immunofluorescence became strong
9
10
11 197 after centrifugation. This phenomenon may be due to better accessibility for
12
13 198 visualization of the labeling after detachment of the PV from the cortex. Ten and 30 min
14
15
16 199 after centrifugation, symbiotic algae were distributed throughout the cell because of
17
18 200 algal relocation. Immunofluorescence of mitochondria was also observed around the
19
20 201 symbiotic algae. These observations suggest that symbiotic algae do not need
21
22 202 trichocysts for their attachment but are colocalized with mitochondria ~~for their~~
23
24 203 ~~attachment~~, as shown by TEM observations (Fig. 1).
25
26
27

28
29 204 Figure 3B shows the immunofluorescence intensity of algae-free *P. bursaria* cells
30
31 205 before (x-bar, M-B), immediately after (x-bar, M-Ia), 10 min (x-bar, M-10 min), and 30
32
33 206 min (x-bar, M-30 min) after centrifugation. There was almost no change in
34
35 207 immunofluorescence intensity, and no significant difference was observed between the
36
37 208 data for each time point. These quantitative data correspond well with the images shown
38
39 209 in Fig. 3A ~~This quantitative data corresponds well with the results of indirect~~
40
41 210 ~~immunofluorescence microscopy, as shown in Fig. 3A.~~
42
43
44
45

46 211

212 **Mitochondrial distribution of host anterior cortex**

51 213 Kodama (2013) investigated the symbiotic algal distribution of 14 strains of *P.*
52
53 214 *bursaria*. As a result, all strains had symbiotic algae at the ventral, dorsal, and posterior
54
55 215 cortexes, and some cells did not have symbiotic algae at the anterior cortex. This
56
57
58 216 phenomenon is not strain-, syngen-, or mating-type specific. Only 35% of the strain
59
60

1
2
3
4
5
6 217 Yad1g1N cells, also used in this experiment, had symbiotic algae at their anterior
7
8 218 cortex. This paper also reported that the artificial trichocyst-discharge experiments by
9
10 219 the treatment of lysozyme clarified that trichocysts of the anterior cortex are difficult to
11
12 220 remove. Although the high-speed centrifugation experiment clearly showed that
13
14 221 trichocysts are unnecessary for algal reattachment, trichocyst-discharge difficulty of the
15
16 222 anterior cortex may be related to the restrict enrichment of the large PVs. It is unclear
17
18 223 why algae have difficulty adhering to the anterior cortex of their hosts. The high-speed
19
20 224 centrifugation experiment clearly showed that trichocysts are unnecessary for algal
21
22 225 reattachment.
23
24
25
26
27

28 226 On the other hand, host mitochondria remained unchanged in localization by high-
29
30 227 speed centrifugation. This indicated that the presence of mitochondria ~~might~~ may be
31
32 228 ~~essential~~ related for algal adhesion. To determine whether the reason for the algal
33
34 229 difficulty in adhering to the host anterior cortex is due to the low mitochondrial density
35
36 230 in this area, we examined whether there were differences in the number and distribution
37
38 231 of mitochondria between cells with and without symbiotic algae in their anterior part
39
40 232 using indirect immunofluorescence microscopy with the monoclonal antibodies against
41
42 233 host mitochondria.
43
44
45
46
47

48 234 Figure 4 shows differential interference contrast microscopy images of *P. bursaria*
49
50 235 cells with (Algae+) and without (Algae-) symbiotic algae in the anterior end and
51
52 236 immunofluorescence images of those cells using mitochondrial monoclonal antibodies.
53
54 237 Note that mitochondria are present in the anterior end without symbiotic algae as well
55
56
57
58
59
60

1
2
3
4
5
6 238 as in the anterior end with symbiotic algae. This observation shows that not all areas

7
8 239 with mitochondria had algae, but there was a localization bias within the host cell.

9
10
11 240

12
13 241 **Discussion**

14
15
16 242 Localization of endosymbionts near the host cell cortex is a universal phenomenon; the

17
18 243 same phenomenon has been observed in other ciliate -algae or -cyanobacteria

19
20 244 endosymbionts, such as *Mayorella viridis*, *Coleps hirtus*, *Coleps spetai*, *Frontonia*

21
22 245 *leucas*, *Malacophrys sphagni*, *Ophrydium versatile*, *Vorticella* sp., *Climacostomum*

23
24 246 *virens*, *Euplotes daidaleos*, *Halteria bifurcata*, *Stentor polymorphus*, and *Stentor niger*

25
26 247 (Reisser 1986). Furthermore, the ability to adjust the intracellular symbiont position is

27
28 248 likely an important means of optimizing carbon production (Petrou *et al.* 2017).

29
30 249 A previous study showed that the infectivity of *Chlorella* species in *P. bursaria* is

31
32 250 based on their ability to localize beneath the host cell membrane after escaping from the

33
34 251 host digestive vacuole during the early infection process (Kodama and Fujishima 2007).

35
36 252 Furthermore, this algal attachment may be related to the avoidance of host lysosomal

37
38 253 digestion or fusion because algal digestion when the symbiotic algae are attached

39
40 254 beneath the host cell cortex has not been observed (Kodama and Fujishima unpubl.

41
42 255 data).

43
44 256 Acidosomes are organelles responsible for the acidification of DVs before

45
46 257 lysosomal fusion and are distributed throughout the cell (Allen 1993; Kodama 2013).

47
48 258 High-speed centrifugation can also induce the rapid accumulation of host acidosomes

49
50 259 and lysosomes at the anterior end of *Paramecium*. Within 10 min of centrifugation, the

1
2
3
4
5
6 260 accumulated vesicles recover their original positions by host rapid cytoplasmic
7
8 261 streaming (Kodama 2013). This observation showed that although acidosomes and
9
10 262 lysosomes were dislocated by centrifugation, the mitochondria remained unchanged.
11
12
13 263 This is probably because acidosomes and lysosomes undergo cytoplasmic streaming,
14
15
16 264 but mitochondria do not.

17
18 265 Figure 5 shows a schematic representation of the algal attachment mechanism
19
20
21 266 beneath the host cell cortex after dislocation via high-speed centrifugation. After the
22
23 267 high-speed centrifugation, almost all trichocysts are released out of the cell. The
24
25
26 268 discharged trichocysts were regenerate from the endoplasmic reticulum, pass through
27
28
29 269 the Golgi apparatus, and undergo a maturation process (pretrichocysts) before mature
30
31 270 trichocysts are delivered to the cell membrane (Plattner 2017). Pretrichocysts contain a
32
33
34 271 growing mass of electron dense secretory material. The pretrichocysts then elongate
35
36 272 while their luminal space is progressively filled with crystallizing secretory materials
37
38
39 273 (Garreau De Loubresse 1993). Because several hours are required for regeneration after
40
41 274 trichocyst discharge (Harumoto 2002), no trichocysts are observed in the cells after
42
43
44 275 algal reattachment. This representation can be defined by obtaining monoclonal
45
46 276 antibodies against both mitochondria and trichocysts. To the best of our knowledge, this
47
48
49 277 is the first report showing the distribution of host mitochondria and trichocysts during
50
51 278 algal relocalization beneath the host cell cortex.

52
53 279 Fast cytoplasmic streaming after high-speed centrifugation of *P. bursaria* is the
54
55
56 280 driving force for algal relocalization. The characteristic feature of rotational cytoplasmic
57
58
59 281 streaming in the genus *Paramecium* (*P. aurelia*, *P. caudatum*, *P. bursaria*, *P.*

1
2
3
4
5
6 282 *multimicronucleatum*, and *P. calkinsi*) is that the pattern of its route and direction
7
8 283 remain constant (Sikora 1981). Various factors, such as cell division, conjugation,
9
10 284 temperature, and osmotic pressure, are known to affect cytoplasmic streaming in
11
12 285 *Paramecium* (Sikora 1981), but the detachment of the symbiotic algae by high-speed
13
14 286 centrifugation is also a factor. In *P. bursaria*–*Chlorella* endosymbiosis, their cell cycle
15
16 287 pace is guaranteed to be synchronous (Takahashi 2016). Takahashi *et al.* (2007) found
17
18 288 that *P. bursaria* controls the proliferation of endosymbiotic algae through host-cell
19
20 289 cycle-dependent cytoplasmic streaming. Thus, host cytoplasmic streaming plays a major
21
22 290 role in maintaining the number and cellular localization of symbiotic algae in *P.*
23
24 291 *bursaria* cells.

25
26 292 The mitochondria of the host *P. bursaria* are close to the PV membrane (Fig. 1),
27
28 293 and their number and function are altered by the presence or absence of symbiotic algae
29
30 294 (Kodama and Fujishima 2022). Therefore, host mitochondria and symbiotic algae are
31
32 295 expected to be strongly associated with each other. We assumed that the presence of
33
34 296 mitochondria was essential for the existence of symbiotic algae; however, the results in
35
36 297 Fig. 4 contradict this expectation. Mitochondria and endoplasmic reticulum (ER) are
37
38 298 contact at ER-Mitochondria Contact Sites (EMCS)s. Lipids and Ca²⁺ synthesized in the
39
40 299 ER are transported to mitochondria through the EMCs (Kornmann *et al.* 2009; Rizzuto
41
42 300 *et al.* 1998). Furthermore, EMCs is also important in reactive oxygen species (ROS)
43
44 301 production (Booth *et al.* 2016) and autophagosome formation (Hamasaki *et al.* 2013).
45
46 302 Song *et al.* (2017) reported that the host endoplasmic reticulumER is also involved in
47
48 303 organizing intracellular algal symbiosis in the cytoplasm. The relationship between host
49
50
51
52
53
54
55
56
57
58
59
60

1
2
3
4
5
6 304 mitochondria and symbiotic algae and the relationship between other cellular
7
8 305 organelles, including the host endoplasmic reticulum, should be examined in detail in
9
10
11 306 future studies.

12
13 307 The exposure of *P. bursaria* cells to ultraviolet and photosynthetically active
14
15
16 308 radiation is known to induce symbiotic algal dislocation, moving to the posterior cell
17
18 309 region (Summerer *et al.* 2009). The displaced algae relocate to a "normal" distribution
19
20
21 310 when host cells are transferred to a medium without ultraviolet radiation conditions. The
22
23 311 mechanisms of algal displacement and relocation remain unclear (Summerer *et al.*
24
25
26 312 2009). A dislocation of *Chlorella* symbionts has also been described in the ciliate
27
28 313 *Pelagodileptus trachelioides* (Butkay 2004). Although this phenomenon appears to be a
29
30
31 314 stress reaction followed by host cytolysis, the details remain unknown. The method of
32
33 315 artificial algal detachment and subsequent rapid reattachment by high-speed
34
35
36 316 centrifugation of algae-bearing *P. bursaria* may contribute to understanding the
37
38 317 mechanism of symbiotic algal localization in a variety of host species.

39
40
41 318 Although the mechanism of PV localization beneath the host cell cortex is still
42
43 319 unknown, the docking or distribution mechanism of mitochondria and uninserted
44
45
46 320 trichocysts in other ciliate species, such as *Paramecium tetraurelia*, have been reported.
47
48 321 Aufderheide (1977) reported that, in the subcortical regions of *P. tetraurelia*,
49
50
51 322 mitochondria and uninserted (undocked) trichocysts display saltatory motility with
52
53 323 individual characteristics, making them distinguishable from each other and from
54
55
56 324 cellular cyclosis. The saltatory motion of trichocysts is implicated as a means of
57
58 325 transporting new trichocysts from the cytoplasm to their ultimate location in the cellular
59
60

1
2
3
4
5
6 326 cortex. In addition, the saltatory motion may be a factor in the intracellular distribution
7
8 327 of the mitochondria. The mechanism of trichocyst docking at the *Paramecium* cell
9
10 328 membrane has been reported in detail (Plattner 2017; Plattner *et al.* 1982). Before
11
12 329 trichocyst docking, mature trichocysts move through the subcortical region by cyclosis
13
14 330 (Sikora 1981). Then, they approach, in a saltatory manner, tip first, a docking site at the
15
16 331 cell membrane (Aufderheide 1978); Thereby, trichocysts are guided by the same
17
18 332 “hand.” This entity is a microtubule emanating from a nearby ciliary basal body
19
20 333 (Plattner *et al.* 1982), which always is situated ~ 0.5 to 1 μm from a docking site proper.
21
22 334 Petrou *et al.* (2017) found that the intracellular algal symbionts within the large
23
24 335 photosymbiotic foraminifera *Marginopora vertebralis* exhibit phototactic behavior and
25
26 336 the phototactic movement of the symbionts is accomplished by the host through rapid
27
28 337 actin-mediated relocation of the symbionts deeper into the cavities within the calcite
29
30 338 skeletons. Thus, the host cytoskeleton plays a major role in the migration of host
31
32 339 cytosolic organelles and symbiotic algae. In order to study the involvement of the
33
34 340 cytoskeleton in the process of PV localization, the cell cortex of algae-bearing *P.*
35
36 341 *bursaria* cells was observed in detail by TEM. However, no cytoskeletal structure that
37
38 342 seems to be related to PV localization has been found until now (Kodama unpub. Data).
39
40 343 Adhesion just below the host cell surface is a universal phenomenon found not
41
42 344 only in cell organelles and symbiotic algae but also in pathogenic bacteria. Protists are
43
44 345 potential natural hosts of various bacterial species, including pathogens. *Paramecium*
45
46 346 *caudatum* could be a natural reservoir of *Legionella pneumophila* (Watanabe *et al.*
47
48 347 2016), and *P. bursaria* could be a potential host of *Francisella novicida* (Watanabe *et*
49
50
51
52
53
54
55
56
57
58
59
60

1
2
3
4
5 348 *al.* 2022). *Francisella novicida* is a facultative intracellular pathogen and a causative
6
7 349 agent of tularemia (Ellis *et al.* 2002). Recently, Watanabe *et al.* (2022) found that *F.*
8
9 350 *novicida* (strain U112) cells, which were wrapped with the host DV membrane,
10
11 351 localized beneath the host cell cortex. This indicates that both symbiotic algae and
12
13 352 bacteria can attach beneath the host cell cortex. Therefore, elucidation of the mechanism
14
15 353 of localization beneath the host cell cortex using *P. bursaria* is expected to contribute to
16
17 354 the development of not only symbiotic biology but also infectious disease research.
18
19
20
21
22
23
24

25 356 **Funding:**

26
27 357 This work was supported by a Grant-in-Aid for Scientific Research (C) (Grant number
28
29 358 20K06768) from the Japan Society for the Promotion of Science (JSPS) and the
30
31 359 Institute for Fermentation (IFO; Osaka, Japan) to YK and Tokubetsukeihi from MEXT
32
33 360 to MF. The authors thank the faculty of Life and Environmental Sciences at Shimane
34
35 361 University for financial support in publishing this report.
36
37
38
39
40
41

42 363 **Acknowledgments:**

43
44 364 *Paramecium* was provided by the NBRP *Paramecium* Laboratory, Yamaguchi
45
46 365 University, with support, in part, by the NBRP of the Ministry of Education, Culture,
47
48 366 Sports, Science, and Technology (MEXT). We would like to thank Editage
49
50 367 (www.editage.com) for English language editing.
51
52
53
54
55
56
57
58
59
60

1
2
3
4
5
6 **369 Ethics declarations:**

7
8 **370 Consent to participate**

9
10
11 All authors have their consent to participate.

12
13 **372 Consent to publish**

14
15
16 All authors have their consent to publish their work.

17
18 **374 Contributions**

19
20
21 Conceived and designed the experiments: YK and MF. Performed the experiments: YK.

22
23
24 Wrote the paper: YK and MF.

25
26
27 377

28
29 **378 Ethical approval**

30
31
32 Not applicable.

33
34
35 Competing interests. The authors declare no competing interests.

36
37
38 381

39
40 **382 References**

41
42
43 **383** Adoutte A. Exocytosis: Biogenesis, Transport and Secretion of Trichocysts. In: Görtz
44
45 **384** HD (ed). *Paramecium*. Springer: Berlin, Heidelberg, 1988, 325–62.

46
47
48 385

49
50
51 **386** Allen RD. Acidosomes: recipients of multiple sources of membrane and cargo during
52
53 **387** development and maturation. *J Cell Sci* 1993;**106**:411–22.

54
55
56 388

57
58
59 **389** Alberts D, Wiessner W. Nitrogen nutrition of endosymbiotic *Chlorella* spec. *Endocyt*
60
Cell Res 1985;**1**:55–64.

391

- 1
2
3
4
5 392 Albers D, Reisser W, Wiessner W. Studies on the nitrogen supply of endosymbiotic
6
7 393 chlorellae in green *Paramecium bursaria*. *Plant Sci Lett* 1982;**25**:85–90.
8
9
10 394
11
12 395 Aufderheide KJ. Saltatory motility of uninserted trichocysts and mitochondria in
13
14 396 *Paramecium tetraurelia*. *Science* 1977;**198**:299–300.
15
16
17 397
18
19 398 Aufderheide KJ. Motility events of trichocyst insertion in *Paramecium tetraurelia*. *J*
20
21 399 *Protozool* 1978;**25**:362–65.
22
23
24 400
25
26 401 Blanc G, Duncan G, Agarkova I *et al.* The *Chlorella variabilis* NC64A genome reveals
27
28 402 adaptation to photosynthesis, coevolution with viruses, and cryptic sex. *The Plant Cell*
29
30 403 2010;**22**:2943–55.
31
32
33 404
34
35 405
36
37 406 Booth DM, Enyedi B, Geiszt M *et al.* Redox Nanodomains Are Induced by and Control
38
39 407 Calcium Signaling at the ER-Mitochondrial Interface. *Mol Cell* 2016;**63**:240–48.
40
41
42 408
43
44 409 Brown JA, Nielsen PJ. Transfer of photosynthetically produced carbohydrate from
45
46 410 endosymbiotic *Chlorellae* to *Paramecium bursaria*. *J Protozool* 1974;**21**:569–70.
47
48
49 411
50
51 412 Butkay M. Beobachtungen an *Pelagodileptus trachelioides* (Ciliophora). *Lauterbornia*
52
53 413 2004;**49**:129–40.
54
55
56 414
57
58
59
60

- 1
2
3
4
5 415 Cheng YH, Liu CFJ, Yu YH *et al.* Genome plasticity in *Paramecium bursaria* revealed
6
7 416 by population genomics. *BMC Biology* 2020;**18**:180.
8
9
10 417
11
12 418 Dryl S. Antigensic transformation in *Paramecium aurelia* after homologous antiserum
13
14 419 treatment during autogamy and conjugation. *J Protozool* 1959;**6**:25.
15
16
17 420
18
19 421 Ellis J, Oyston PC, Green M *et al.* Tularemia. *Clin Microbiol Rev* 2002;**15**:631–46.
20
21 422
22
23 423 Fujishima M, Kodama Y. Endosymbionts in *Paramecium*. *Europ J Protistol*
24
25 424 2012;**48**:124–37.
26
27
28 425
29
30 426 Garreau De Loubresse N. Early steps of the secretory pathway in *Paramecium*:
31
32 ultrastructural, immunocytochemical, and genetic analysis of trichocyst biogenesis. In:
33 427 Plattner H (ed). *Membrane Traffic in Protozoa*, JAI Press, Greenwich (CT, USA),
34
35 428 London (GB), 1993, 27–59.
36
37 429
38
39 430
40
41 431
42
43 432 Gortz HD. Infections of *Paramecium bursaria* with bacteria and yeasts. *J Cell Sci* 1982;
44
45 433 **58**:445–53.
46
47
48 434
49
50 435 Gu FK, Chen L, Ni B, Zhang X. A comparative study on the electron microscopic
51
52 436 enzymo-cytochemistry of *Paramecium bursaria* from light and dark cultures. *Eur J*
53
54 437 *Protistol* 2002;**38**:267–78.
55
56
57 438
58
59
60

1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17
18
19
20
21
22
23
24
25
26
27
28
29
30
31
32
33
34
35
36
37
38
39
40
41
42
43
44
45
46
47
48
49
50
51
52
53
54
55
56
57
58
59
60
439

440 [Hamasaki M, Furuta N, Matsuda A et al. Autophagosomes form at ER-mitochondria](#)
441 [contact sites. *Nature* 2013;**495**:389–93.](#)

442

443 [Harumoto T. Defensive function of trichocysts in *Paramecium*. *Jpn J Protozool*](#)
444 [2002;**35**:125–33.](#)

445

446 Karakashian SJ, Rudzinska MA. Inhibition of lysosomal fusion with symbiont-
447 containing vacuoles in *Paramecium bursaria*. *Exp Cell Res* 1981;**131**:387–93.

448

449 Kodama Y. Localization of attachment area of the symbiotic *Chlorella variabilis* of the
450 ciliate *Paramecium bursaria* during the algal removal and reinfection. *Symbiosis*
451 2013;**60**:25–36.

452 Kodama Y, Fujishima M. Infectivity of *Chlorella* species for the ciliate *Paramecium*
453 *bursaria* is not based on sugar residues of their cell wall components, but on their
454 ability to localize beneath the host cell membrane after escaping from the host digestive
455 vacuole in the early infection process. *Protoplasma* 2007;**231**:55–63.

456

457 Kodama Y, Fujishima M. Localization of perialgal vacuoles beneath the host cell
458 surface is not a prerequisite phenomenon for protection from the host's lysosomal fusion
459 in the ciliate *Paramecium bursaria*. *Protist* 2009;**160**:319–29.

460

461 Kodama Y, Fujishima M. Secondary symbiosis between *Paramecium* and *Chlorella*
462 cells. *Int Rev Cell Mol Biol* 2010;**279**:33–77.

- 1
2
3
4
5 463
6
7
8 464 Kodama Y, Fujishima M. Endosymbiosis of *Chlorella* species to the ciliate
9
10 465 *Paramecium bursaria* alters the distribution of the host's trichocysts beneath the host
11
12 466 cell cortex. *Protoplasma* 2011;**248**:325–37.
13
14 467
15
16
17 468 Kodama Y, Fujishima M. Synchronous induction of detachment and reattachment of
18
19 469 symbiotic *Chlorella* spp. from the cell cortex of the host *Paramecium bursaria*. *Protist*
20
21 470 2013;**164**:660–72.
22
23
24 471
25
26 472 Kodama Y, Fujishima M. Endosymbiotic *Chlorella variabilis* reduces mitochondrial
27
28 473 number in the ciliate *Paramecium bursaria*. *Sci Rep* 2022;**12**:8216.
29
30 474
31
32
33 475 Kodama Y, Inouye I, Fujishima M. Symbiotic *Chlorella vulgaris* of the ciliate
34
35 476 *Paramecium bursaria* plays an important role in maintaining perialgal vacuole
36
37 477 membrane functions. *Protist* 2011;**162**:288–303.
38
39
40 478
41
42 479 [Kornmann B, Currie E, Collins SR et al. An ER-mitochondria tethering complex](#)
43
44 480 [revealed by a synthetic biology screen. *Science* 2009;**325**:477–81.](#)
45
46
47 481
48
49 482 Petrou K, Ralph PJ, Nielsen DA. A novel mechanism for host-mediated photoprotection
50
51 483 in endosymbiotic foraminifera. *ISME J* 2017;**11**:453–62.
52
53
54 484
55
56
57
58
59
60

- 1
2
3
4
5 485 Plattner H. Trichocysts-*Paramecium*'s Projectile-like Secretary Organelles: Reappraisal
6
7 486 of their Biogenesis, Composition, Intracellular Transport, and Possible Functions. *J*
8
9 487 *Eukaryot Microbiol* 2017;**64**:106–33.
10
11
12 488
13
14 489 Plattner H, Westphal C, Tiggemann R. Cytoskeleton secretory vesicle interactions
15
16 490 during the docking of secretory vesicles at the cell membrane in *Paramecium*
17
18 491 *tetraurelia* cells. *J Cell Biol* 1982;**92**:368–77.
19
20
21 492
22
23 493 Reisser W. Endosymbiotic associations of freshwater protozoa and algae. In: Corliss
24
25 494 JO, Patterson DJ (eds). *Progress in Protistology*, Biopress Ltd, Bristol, 1986, 195–214.
26
27
28 495
29
30 496
31
32 497 Rizzuto R, Pinton P, Carrington W et al. Close contacts with the endoplasmic reticulum
33
34 498 as determinants of mitochondrial Ca²⁺ responses. *Science* 1998;**280**:1763–66.
35
36
37 499
38
39 500 Sikora J. Cytoplasmic streaming in *Paramecium*. *Protoplasma* 1981;**109**:57–77.
40
41
42 501
43
44 502 Song C, Murata K, Suzaki T. Intracellular symbiosis of algae with possible involvement
45
46 503 of mitochondrial dynamics. *Sci Rep* 2017;**7**:1221.
47
48
49 504
50
51 505
52
53 506 Summerer M, Sonntag B, Hörtnagl P et al. Symbiotic ciliates receive protection against
54
55 507 UV damage from their algae: A test with *Paramecium bursaria* and *Chlorella*. *Protist*
56
57 508 2009;**160**:233–43.
58
59
60

- 1
2
3
4
5 509
6
7
8 510 Takahashi T. Simultaneous Evaluation of Life Cycle Dynamics between a Host
9
10 511 *Paramecium* and the Endosymbionts of *Paramecium bursaria* Using Capillary Flow
11
12 512 Cytometry. *Sci Rep* 2016;**6**:31638.
13
14 513
15
16 514 Takahashi T, Shirai Y, Kosaka T *et al.* Arrest of Cytoplasmic Streaming Induces Algal
17
18 515 Proliferation in Green *Paramecia*. *PLoS ONE* 2007;**12**:e1352.
19
20 516
21
22 517 Tsukii Y, Harumoto T, Yazaki K. Evidence for a viral macro- nuclear endosymbiont in
23
24 518 *Paramecium caudatum*. *J Euk Microbiol* 1995;**42**:109–15.
25
26 519
27
28 520 Reisser W. The metabolic interactions between *Paramecium bursaria* Ehrbg. and
29
30 521 *Chlorella* spec. in the *Paramecium bursaria*-symbiosis. II. Symbiosis-specific
31
32 522 properties of the physiology and the cytology of the symbiotic unit and their regulation
33
34 523 (author's transl). *Arch Microbiol* 1976;**111**:161–70.
35
36 524
37
38 525 Reisser W. The metabolic interactions between *Paramecium bursaria* Ehrbg. and
39
40 526 *Chlorella* spec. in the *Paramecium bursaria*-symbiosis. III. The influence of different
41
42 527 CO₂-concentrations and of glucose on the photosynthetic and respiratory of the
43
44 528 symbiotic unit. *Arch Microbiol* 1980;**125**:291–93.
45
46 529
47
48 530 Reisser W. Endosymbiotic associations of freshwater protozoa and algae. *Prog Protistol*
49
50 531 1986;**1**:195–214.
51
52 532
53
54
55
56
57
58
59
60

- 1
2
3
4
5 533 Reynolds ES. The use of lead citrate at high pH as an electron-opaque stain in electron
6
7 534 microscopy. *J Cell Biol* 1963;**17**:208–12.
8
9
10 535
11
12 536 Spurr AR. A low viscosity epoxy resin embedding medium for electron microscopy. *J*
13
14 537 *Ultrastruct Res* 1969;**26**:31–43.
15
16
17 538
18
19 539
20
21 540 Watanabe K, Nakao R, Fujishima M *et al.* Ciliate *Paramecium* is a natural reservoir of
22
23 541 *Legionella pneumophila*. *Sci Rep* 2016;**6**:24322.
24
25
26 542
27
28 543 Watanabe K, Motonaga A, Tachibana M *et al.* *Francisella novicida* can
29
30 544 utilize *Paramecium bursaria* as its potential host. *Envl Microbiol Rep* 2022;**14**:50–9.
31
32
33
34
35
36
37
38
39
40
41
42
43
44
45
46
47
48
49
50
51
52
53
54
55
56
57
58
59
60