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The ciliate *Paramecium bursaria* allows budding of symbiotic *Chlorella variabilis* cells singly from the digestive vacuole membrane into the cytoplasm during algal reinfection

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Abstract

The ciliate *Paramecium bursaria* harbors several hundred symbiotic *Chlorella* spp. cells in the cytoplasm. Algal re-endosymbiosis can be artificially induced using alga-removed *P. bursaria*. During algal re-endosymbiosis, algae ingested into the host digestive vacuoles (DVs) avoid digestion by the host lysosomal enzymes and then escape into the cytoplasm by budding off of the DV membrane. The budded alga-enclosing DV membrane then differentiates into the symbiosome or perialgal vacuole (PV) membrane and is localized beneath the host cell cortex. In this study, we determined whether the PV membrane has the ability to recognize the symbiotic alga singly by eliminating other small microspheres in the same DV. To clarify the accuracy of the budding process, we mixed fluorescent-labeled microspheres of diameter 0.20 μm with isolated symbiotic algae during algal re-endosymbiosis. No fluorescence was observed from the PV membrane, as expected, and the budding DVs that enclosed both undigested and digested algae. Additionally, the algal re-endosymbiosis rate was significantly reduced in

the presence of microspheres. These observations showed that the host *P. bursaria* allowed budding of the algae singly from the membranes of DVs without microspheres and this process required close contact between the DV membrane and the algal cell wall.

Introduction

Many non-photosynthetic species of alveolata, katablepharidophyta, rhizeria, and metazoa are capable of hosting algal endosymbionts or their organelles through adaptations to the host phagocytic process (Johnson 2011; Stoecker et al. 2009). The ciliate *Paramecium bursaria* can harbor green algal *Chlorella* sp. cells in its cytoplasm. Both cells share a mutualistic association, where they benefit from each other by living together. Symbiotic algae provide the host with photosynthetic products, such as oxygen and maltose (Brown and Nielsen 1974), whereas the host *P. bursaria* provides the symbiotic algae with nitrogen and carbon sources required for photosynthesis (Albers and Wiessner 1985). The population size of symbiotic algae in the host cell is maintained at a constant level under growth in the presence of light and nutrients (Iwai et al. 2016). Symbiotic algal digestion mechanisms permit host *P. bursaria* cells to survive for a longer time under conditions of starvation (Kodama and Miyazaki 2021). Although the relationship between *P. bursaria* and symbiotic algae constitutes mutual symbiosis, both cells can be cultured alone. By cultivating alga-bearing *P. bursaria* cells under constantly dark conditions or treatment with a photosynthesis inhibitor (Reisser 1976) or a protein synthesis inhibitor (Weis 1984), symbiotic *Chlorella* spp. can be artificially removed from the cells. Algal re-endosymbiosis can be easily induced by mixing isolated symbiotic algae from alga-bearing and alga-free (i.e. alga-removed) *P. bursaria* (Kodama and Fujishima 2005; Siegel and Karakashian 1959). Therefore, *P. bursaria* and their symbiotic *Chlorella* spp. are used as model organisms to clarify the establishment process of animal (i.e. *P. bursaria*) and plant cell (i.e. *Chlorella* spp.) endosymbiosis. Transcriptomic and genomic analyses of *P. bursaria* have been conducted recently and the genetic basis of the establishment of endosymbiosis in this organism has been elucidated (Cheng et al. 2020; He et al. 2019; Kodama et al. 2014).

During re-endosymbiosis of *Chlorella* sp. in alga-free *P. bursaria* cells, the algae are taken up into the *P. bursaria* cells by phagocytosis and enveloped by the digestive vacuole (DV) membrane. Thereafter, both acidosomes and lysosomes that contain digestive enzymes fuse. It has been revealed that four important cytological phenomena occur during the algal re-endosymbiosis process (Fujishima and

59 Kodama 2012; Kodama and Fujishima 2010). First, some algae enclosed by the DV membrane avoid
60 digestion by showing resistance to the host lysosomal digestive enzymes. Then, the alga buds off of the
61 DV membrane and escapes into the host cytoplasm. Subsequently, the DV membrane surrounds the
62 budded undigested alga and differentiates into the perialgal vacuole (PV) membrane, thereby blocking
63 host lysosomal fusion. As the definition of the term PV membrane represents, whether the membrane
64 contains a single or multiple *Chlorella*, and whether the membrane contains anything other than *Chlorella*
65 is important for distinguishing between DV and PV membranes. Finally, the alga wrapped by the PV
66 membrane is localized beneath the host cell cortex. The above four steps are considered indispensable for
67 re-endosymbiosis, but their underlying mechanisms have not yet been elucidated.

68 Both the living and digested algae escape into the cytoplasm by budding off of the DV
69 membranes in *P. bursaria* (Kodama and Fujishima 2005). This result showed that the algae did not need
70 to be alive to bud off of the DV membrane. Both living and boiled *Saccharomyces cerevisiae* cells and
71 polystyrene latex beads of diameter ≥3 μm could also bud off of the DV membranes (Kodama and
72 Fujishima 2012). However, budding was not observed when India ink, polystyrene latex beads of
73 diameter 0.81 μm, or food bacteria (*Klebsiella pneumoniae*) were ingested into the DVs (Kodama and
74 Fujishima 2005). These results suggested that *P. bursaria* could recognize the diameter of the DV
75 contents and cells of diameter ≥3 μm could escape into the cytoplasm by budding off of the DV
76 membrane. Although the several phagocytosis experiments using microspheres have been conducted in
77 *Paramecium* spp., these experiments have been carried out by using microspheres alone, and there were
78 no reports using microspheres and algae together (Fok et al. 1988; Kodama and Fujishima 2012; Wiejak
79 et al. 2003). Therefore, whether algal cells can escape into the cytoplasm singly by budding off of the DV
80 membrane remains unclear.

81 Not only the symbiotic relationship between *P. bursaria* and *Chlorell*a spp., protection of the
82 symbionts or parasites from host lysosomal fusion is also observed in ciliate *Climacostomum virens*
83 (Reisser 1984), cnidarian *Hydra viridis* (Hohman et al. 1982; O'Brien 1982), and apicomplexan
84 *Toxoplasma gondii* (Scholtyseck and Piekarski 1965). Also, the endosymbiotic X-bacteria in *Amoeba*
85 *proteus* (Ahn et al. 1990), symbiotic *Chlorella variabilis* in *Stentor pyriformis* (Hoshina et al. 2021), the
86 dinoflagellate *Symbiodinium microadriaticum* (Fitt and Trench 1983), and *Mycobacterium tuberculosis*
87 (Deretic and Fratti 1999; Sinai and Joiner 1997) are enclosed in a vacuole, forming so-called

88 ‘‘symbiosomes’’ derived from the host DV membrane. Although PV membranes are very important for
89 the establishment and maintenance of endosymbiosis, not much is known about the features. (1) The PV
90 membrane encloses a single algal cell (Gu et al. 2002; Karakashian and Rudzinska 1981); (2) The PV
91 membrane is closely appose to the algal cell wall and the PV membrane contacts to the host
92 mitochondrial membrane directly (Song et al. 2016); (3) The PV diameter does not vary greatly, except
93 during the division of the enclosed symbiotic alga (Reisser 1992); (4) The PV does not participate in
94 cyclosis, but it localizes immediately beneath the host cell cortex (Kodama and Fujishima 2005; Reisser
95 1986); (5) Particle density and its distribution of the PV membrane show few signs hinting at any
96 endocytotic or exocytotic activity, which can be observed in the DV membrane (Meier et al. 1984). To
97 examine the PV membrane features during the early algal re-endosymbiosis process to the algae-removed
98 *P. bursaria* cells, we used fluorescent-labeled latex beads (Fluoresbrite® YG Microspheres) of diameter
99 0.2 μm. By using the fluorescent microspheres, we succeeded to determine whether the algae could
100 escape into the cytoplasm singly and whether the host *P. bursaria* cell possessed the ability to distinguish
101 the beads from the symbiotic algae based on their diameters. In particular, we observed the presence or
102 absence of fluorescence of the microspheres in 1) the membrane of the alga that escaped into the host
103 cytoplasm, 2) the membrane that surrounded the escaped alga, and 3) the PV membrane attached beneath
104 the host cell cortex as observed by a differential interference contrast (DIC) and fluorescence microscope.
105 Additionally, algal re-endosymbiosis experiments were performed with or without microspheres to
106 determine and measure any changes in the re-endosymbiosis rates.
107
108 **Materials and methods**
109 **Cultivation of symbiotic *Chlorella* sp. -bearing and -free *P. bursaria* cells**
110 The symbiotic *Chlorella* sp.-free (alga-removed) *P. bursaria* strain Yad1w and the *Chlorella* sp.-bearing
111 *P. bursaria* strain Yad1g1N were provided by Symbiosis Laboratory, Yamaguchi University (Japan) with
112 partial support of the National BioResource Project of the Japan Agency for Medical Research and
113 Development (http://nbrpcms.nig.ac.jp/paramecium/?lang=en). *Paramecium* cells were then cultured in
114 red pea (*Pisum sativum*) extract culture medium (Tsukii et al. 1995) in modified Dryl’s solution (MDS)
115 (Dryl 1959) (KH_2PO_4 was used instead of $NaH_2PO_4 \cdot 2H_2O$), which was inoculated with a
116 non-pathogenic *K. pneumoniae* strain (strain 6081) 1 day before use (Fujishima et al. 1990). In ordinary

cultures, several hundred cells were inoculated into 2 mL aliquots of the culture medium in test tubes. Subsequently, 2 mL aliquots of fresh culture medium were added every day for 12 days. All cultures were used in experiments during the early stationary growth phase 1 day after the final feeding and cultivated at 25 ± 1℃. Alga-bearing cells were cultured under fluorescent lighting at 20–30 μmol photons $m^{-2}s^{-1}$ using an incandescent lamp.

Isolation of symbiotic algae from alga-bearing *P. bursaria*

Yad1g1N cells from cultures (300 mL) were strained through two layers of KimWipes to remove gross debris. Cells were then transferred to a centrifuge tube equipped with a nylon mesh of a pore size of 15 μm and filtered through it. *Paramecium* cells were washed with fresh MDS (50 mL), harvested using a hand-operated centrifuge (UKG-2, Uchida Rikakiki Tokyo Japan), and resuspended in MDS (1 mL). The sedimented cells were suspended in 0.1 mM phenylmethylsulphonyl fluoride (Sigma-Aldrich, St. Louis, MO, USA)-containing MDS (1 mL) and homogenized by hand in a Teflon homogenizer on ice. Homogenates were then transferred to a centrifuge tube equipped with a nylon mesh of a pore size of 15 μm. The filtrate was transferred to 1.5 mL tubes, washed in MDS (1.5 mL) thrice by centrifugation at 4500 x g for 1 min, and then reduced to a final volume of 500 μL. The *Chlorella* sp. cell density was calculated using a blood-counting chamber.

Experiments on re-endosymbiosis of isolated symbiotic algae in alga-free *P. bursaria* cells using Fluoresbrite® YG Microspheres

Cells from Yad1w strain cultures (50 mL) were filtered through two layers of KimWipes to remove gross debris and then filtered in centrifuge tubes equipped with a nylon mesh of a pore size of 15 μm. *Paramecium* cells were washed with fresh MDS (50 mL), and the harvested cells were resuspended in MDS (5 mL). *Paramecium* cells (5×10^3 cells/mL) were mixed with isolated symbiotic *Chlorell*a sp. cells (5×10^7 algal cells/mL) and Fluoresbrite® YG Microspheres (diameter, 0.20 μm; 5.68×10^{12} particles/mL; Polysciences, Warrington, PA, USA) in a centrifuge tube (10 mL) at 25 ± 1℃ for 1.5 min to obtain final concentrations of 0.5% or 1.0%. The ciliate-alga-microsphere mixture was transferred to a centrifuge tube equipped with a nylon mesh of a pore size of a 15 μm and filtered through it. *Paramecium* cells were washed with fresh MDS (100 mL), and both the algal cells and microspheres outside the

146 *Paramecium* cells were simultaneously removed through the mesh. The *Paramecium* cells retained in the
147 mesh were transferred to a centrifuge tube (10 mL), resuspended in MDS (1 mL), and then observed
148 under a fluorescent light (1500 lux) at 25 ± 1°C. A 100 μL aliquot of the cell suspension was fixed using
149 an equal volume of 8% (w/v) paraformaldehyde at 10 min, 30 min, 45 min, and 24 h after mixing with the
150 algae, and the cells were observed under a DIC and fluorescence microscope (BX53; Olympus, Tokyo,
151 Japan) equipped with Olympus fluorescence mirror units U-FBNA (excitation 470 to 495 nm, emission
152 510 to 550 nm) for AF 488 and U-FGW (excitation 530 to 550 nm, emission 575 nm) for algal
153 autofluorescence. Images were acquired using the Olympus DP74 (Olympus) and analyzed using the
154 Olympus cellSens Dimension software (Olympus). For some of the re-endosymbiosis experiments, dead
155 algae were used instead of living algae. For this, the algae were first boiled for 10 min using Dry Block
156 Bath (BMS, NY, USA) and then cooled on ice.
157
158 **Results and discussion**
159 **Rigorous budding of the algae from the DV membrane into the cytoplasm**
160 Several algae passed through the host cytopharynx and were pinched off as DVs 10 min after mixing the
161 alga-microsphere mixture with alga-free *P. bursaria* cells (Fig. 1a, D). Several microspheres were
162 enclosed with the algae in the DV(s) (Fig. 1b, D). The presence of microspheres was indicated by green
163 fluorescence (Fig. 1b). Undigested green alga was observed escaping into the cytoplasm by budding off
164 of the DV membrane (Fig. 1c', black arrowhead) 30 min after mixing the alga-microsphere mixture with
165 alga-free *P. bursaria* cells (Fig. 1c, d, c', and d'). Note that the fluorescence of the microspheres did not
166 overlap with the autofluorescence of the alga, as observed by fluorescence microscopy (Fig. 1d', white
167 arrowhead). Both undigested single green alga (Fig. 1e', black arrowhead) and single digested alga (Fig.
168 1e', black arrow) were observed budding off of the DV membrane 45 min after mixing the
169 alga-microsphere mixture with alga-free *P. bursaria* cells (Fig. 1e, f, e', f', and g). Note that the
170 fluorescence of the microspheres did not overlap with the autofluorescence of the undigested algae (Fig.
171 1f', white arrowhead). Autofluorescence of the digested algae was then lost (Fig. 1f'). Additionally,
172 observation of *P. bursaria* in which the algae had begun to bud off of the DV membrane and escape into
173 the cytoplasm revealed *Chlorella*-sized small vesicles and strong green fluorescence of the microspheres
174 in the cytoplasm (data not shown). This indicated that, similar to the symbiotic algae, the microspheres

enveloped by the DV membrane could bud off into the host cytoplasm. At that time, the algae remained in the host DVs, suggesting that the microspheres had aggregated and budded off of the DV membrane before the algae. On establishing re-endosymbiosis, many single green algae were observed (Fig. 1h, black arrowhead) 24 h after mixing the alga-microsphere mixture with alga-free *P. bursaria* cells (Fig. 1h and i). As these algae were localized beneath the host cell cortex, they could be considered to be wrapped by PV membranes. Note that the fluorescence of the microspheres did not overlap with the autofluorescence of the symbiotic algae (Fig. 1i, white arrowhead).

Fig. 2 shows changes in the fluorescence intensity of the microspheres. Strong fluorescence was observed in the DVs at 10, 15, and 30 min. In contrast, fluorescence was not observed from both the membrane that enclosed the alga budding off of the DV membrane into the cytoplasm and the PV membrane.

When boiled (i.e. dead) algae and microspheres were mixed with alga-free *P. bursaria* cells, individual digested algae, similar to living algae, were observed to bud off of the DV membrane (Fig. 3, arrowhead). Note that the fluorescence of the microspheres did not overlap with that of the undigested algae, similar to the symbiotic algae. To our knowledge, during the early algal re-endosymbiosis process, these observations are the first evidence that *P. bursaria* possessed a precise ability to determine whether the DV contents were symbiotic algae or microspheres and whether the algae could singly bud off of the DV membrane. A schematic representation of the above-mentioned results is shown in Fig. 4a.

A previous study showed that microspheres of diameter 0.20 μm did not induce individual budding of the DVs. In contrast, microspheres of diameter 0.80 μm produced DVs of diameter 5–10 μm 3 min after mixing the microspheres with alga-free *P. bursaria* cells; then, the DVs fragmented into vacuoles of diameter 2–5 μm until 3 h after mixing. These observations revealed that induction of DV budding depended on the size of the DV contents (Kodama and Fujishima 2012). In the present study, when a mixture of isolated symbiotic algae and microspheres of diameter 0.2 μm were mixed with alga-free *P. bursaria*, vacuoles that contained not only alga alone, but also solely microspheres, were observed. This suggested that if contents of a certain size, such as *Chlorella* spp. (approx. 2–4 μm), were taken up into one DV, DV membrane constriction might be induced. However, how the algae are singly budded off of the DV membrane in *P. bursaria* remains unclear. It has been revealed that dynamin, a

protein that is ubiquitous in eukaryotes, is involved in phagocytosis of the phagosomal membrane in *P. bursaria* cells, but the detailed mechanism remains unknown (Kodama and Fujishima 2012).

Effect of the microspheres on the re-endosymbiosis rate

Fig. 5 shows the re-endosymbiosis rate of the algae with (final concentrations, 0.05% or 1%) or without microspheres 24 h after mixing the microspheres with alga-free *P. bursaria*. Re-endosymbiosis rate is the percentage of *Paramecium* cells with green algae beneath the host cell cortex (Kodama and Fujishima 2005). More than 200 cells were observed, and similar results were obtained from three independent experiments. The re-endosymbiosis rate of alga-free *P. bursaria* that contained only isolated symbiotic algae was about 40%, whereas that of alga-free *P. bursaria* that contained microspheres at both final concentrations was significantly reduced to about 10%. A schematic representation of the above-mentioned results is shown in Fig. 4b. Moreover, no effect on the re-endosymbiosis rate of the isolated symbiotic algae enclosed with microspheres of diameter 6.0 μm in the DV, compared with that enclosed without microspheres in the DV, was observed (Kodama and Fujishima unpublished data). It was reported that PV formation probably required algal surface-ciliate membrane contact when ingested algae came into contact with the DV membrane (Reisser 1988). In this case, the algae might be enclosed via a “zipper” mechanism proposed for phagocytic processes in macrophages (Griffin et al. 1976). The zipper mechanism does not operate in nonsymbiotic *Paramecium s*pecies, such as *Paramecium caudatum*, because these algae cannot escape from digestion. Thus, it was speculated that the ability to form PVs, i.e. to respond to a special signaling pattern, might rely on the synthesis of special vesicles that introduced appropriate receptor components into the cytopharynx, DV, or growing PV membranes. Thus, the synthesis of these vesicles, tentatively called PV organizing vesicles, is a special feature that characterizes only PV-forming ciliates, and is absent in *P. caudatum* or *Paramecium tetraurelia* (Reisser 1988). The results of our study clearly support these preceding discussions, because it was speculated that host *P. bursaria* might not be able to receive PV formation-related signals due to the trapping of the small-diameter microspheres between the DV membrane and the algal cell wall (Fig. 4c). Therefore, the number of algae budding off of the DV membrane would decrease, and as a result, a significant decrease in the re-endosymbiosis rate was predicted.

231 It has long been speculated that lectins can be involved in symbiotic algal recognition process in
232 cnidarians (Jimbo and Yamashita 2018). In the endosymbiosis between freshwater *Hydra viridis* and
233 *Chlorella*-like algae, it has been reported that concanavalin A (Con A) completely inhibits reassociation
234 of algae harvested from green hydra to bleached hydra, whereas WGA, ricin, and *Lens culinaris* lectins
235 also inhibit this process, but not at the same level. Lin et al. (2000) reported that, in the endosymbiosis
236 between the sea anemone, *Aiptasia pulchella,* and zooxanthellae, masking of the surface of the
237 zooxanthella with four types of lections (Con A; *Limulus polyphemus* agglutinin, LPA; *Phaseolus*
238 *vulgaris* erythroagglutinin, PHA-E; *Triticum vulgaris* agglutinin, wheat germ, WGA) induced a
239 significant decline in the algal infection rate in bleached adult host cells. Additionally, in the
240 endosymbiosis between *Fungia scutaria* and their endosymbiotic dinoflagellate algae, inhibition of cell
241 surface glycans of the symbiotic algae by FITC- Con A or FITC-Jac resulted in decreased algal
242 reinfection of *F. scutaria* larvae (Wood-Charlson et al. 2006). In *P. bursaria*, Reisser et al. (1982)
243 reported that ConA and *Ricinus communis* lectin prevented uptake of *Chlorella* spp. cells by the host
244 *Paramecium*. Unlike their results, Kodama and Fujishima (2007) confirmed that labeling of their cell
245 walls with Alexa Fluor 488-conjugated WGA, GS-II, or Con A showed no relationship between
246 infectivity and staining with these lectins. Thus, symbiotic algal recognition process in *P. bursaria* still
247 remains unknown.
248
249 **Conclusions**
250 This study reveals the following two important features of PV membranes though the molecular
251 mechanism is unknown. First, when the algal escaping into the host cytoplasm, the membrane that is
252 about to differentiate into a PV membrane has the ability to recognize the symbiotic alga singly by
253 eliminating other small particles in the same DV. Second, for the establishment of algal endosymbiosis, it
254 requires close contact between the DV membrane and the algal cell wall. The close contact between PV
255 membrane and symbiotic alga has known, but about the algal re-endosymbiosis process was not well
256 known until this experiment. It is necessary to elucidate the detailed molecular mechanism of DV
257 membrane constriction by identifying genes involved in membrane constriction and budding using the
258 genome information of *P. bursaria* in future studies.
259

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Conflict of interest The authors declare that they have no conflict of interest.

Code availability Not applicable

Author contributions Yuuki Kodama and Haruka Sumita conceived, designed, and performed the experiments. Yuuki Kodama wrote the manuscript.

Ethics approval Not applicable

Consent to participate Not applicable

Consent for publication Not applicable

Figure legends

Fig. 1. Photomicrographs of alga-free *Paramecium bursaria* mixed with the alga–microsphere mixture. **a**, **c**, **e**, and **h** show the differential interference contrast photomicrographs. **b**, **d**, **f**, and **i** show the fluorescence photomicrographs of **a**, **c**, **e**, and **h**, respectively. **c'**, **d'**, **e'**, and **f'** show enlargement of the square area depicted in **c**, **d**, **e**, and **f**, respectively. **g** shows the merged image of **e'** and fluorescence of microspheres. Ma, macronucleus; d, digestive vacuole; Cy, cytoplasm. Images are representative of at least three independent experiments. Microspheres were observed with the algae in the digestive vacuoles (DVs) (**b**) 10 min after the mixing (**a** and **b**). Undigested green alga was observed budding off of the membranes of DVs without microspheres (**c'** and **d'**, arrowhead) 30 min after the mixing (**c**, **d**, **c'**, and **d'**). Undigested green (**e'**, arrowhead) and digested brown (**e'**, arrow) algae were observed after budding off of the membranes of DVs without microspheres 45 min after the mixing (**e**, **f**, **e'**, **f'**, and **g**). On establishing re-endosymbiosis, single green alga was observed beneath the host cell cortex (**h**, arrowhead)

289 24 h after the mixing (**h** and **i**). Fluorescence of the microspheres did not overlap with the
290 autofluorescence of the symbiotic algae (**i**, arrowhead).
291
292 **Fig. 2.** Fluorescence brightness of the microspheres in the digestive vacuoles (DVs) or perialgal vacuoles
293 (PVs) during algal reinfection. Strong fluorescence of the DVs was observed. Fluorescence was not
294 observed from both the membrane that enclosed the budding alga and the PV membrane. Bars in the
295 graphs represent standard deviation. In each category, fluorescence intensity of 12–25 DVs or PVs were
296 analyzed. The photomicrograph under each bar graph shows the representative image of each category.
297
298 **Fig. 3.** Merging of the differential interference contrast photomicrograph and fluorescence of the
299 microspheres. When boiled algae and microspheres were mixed with alga-free *Paramecium bursaria*
300 cells, individual digested algae, similar to living algae, were observed budding off of the digestive
301 vacuole membrane without fluorescence (arrowhead). Ma, macronucleus
302
303 **Fig. 4.** Schematic representation of the results obtained in this study. (**a**) When isolated symbiotic algae
304 were enclosed in the host digestive vacuoles (DVs) with (ii) or without (i) microspheres (yellow small
305 spheres in the figure), both undigested and digested alga could singly bud off of the membranes of DVs
306 with (ii-3) and without (i-3) microspheres. Note that small vacuoles that enclosed only microspheres
307 could also be observed. Boiled algae with microspheres (iii) could also singly bud off of the membranes
308 of DVs without microspheres (iii-3). (**b**) The reinfection rate (i.e. the percentage of the cells with green
309 algae beneath the host cell cortex) of the isolated symbiotic algae mixed with alga-free *Paramecium*
310 *bursaria* cells with microspheres (ii), compared with that of the isolated symbiotic algae mixed with
311 alga-free *P. bursaria* cells without microspheres (i), was significantly reduced. (**c**) When the
312 microspheres were enclosed with isolated symbiotic algae in the DVs (ii), the microspheres were
313 expected to enter the space between the DV membrane and the algal cell wall (ii-2). In such cases, algal
314 budding off of the DV membrane might be prevented (ii-3). Therefore, more algae appeared to bud from
315 the membranes of DVs without microspheres (i-3).
316

Fig. 5. Percentage of the cells with green algae beneath the host cell cortex. The symbiosis rate of alga-free *Paramecium bursaria* after 24 h was examined by mixing the only *Chlorella*-containing cells with the alga-microsphere mixture at final concentrations of 1% and 0.05%. In the presence of beads, the symbiosis rate was reduced. Error bars reflect 90% confidence limits. Asterisks indicate significant differences (Fisher's exact test, $^{**}P < 0.01$).

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