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Symbiotic *Chlorella variabilis* strain, 1 N, can influence the digestive process in the host *Paramecium bursaria* during early infection

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Abstract The association between the ciliate *Paramecium bursaria* and symbiotic *Chlorella* spp. is mutually beneficial. However, this relationship is facultative mutualism because both the host and the symbiotic algae can grow by themselves. This association is easily re-established by mixing the two species together. Following algal mixing, some algae become enclosed in the digestive vacuole membrane of the paramecia to which both acidosomes and lysosomes fuse. To establish endosymbiosis, some algae acquire temporal resistance to the host lysosomal enzymes in the digestive vacuoles. We examined whether the algae influence the differentiation of the host digestive process using LysoSensor staining to evaluate the acidification of the digestive vacuoles. Furthermore, to assess lysosomal fusion with the digestive vacuole, Gomori's staining was conducted. Acidification and lysosomal fusion occurred later in digestive vacuoles containing living algae than in those containing boiled algae or latex spheres. This phenomenon was observed when the living algae were maintained under a constant light condition. These results suggest that the algae release some unknown factor in response to light exposure, and the factor may be associated with the alteration of the host digestive process, indicating that the living algae can influence the host digestive processes during early algal infection.

Keywords *Chlorella* spp. · Digestion · Endosymbiosis · Gomori's staining · LysoSensor · *Paramecium bursaria*

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1 Introduction

Endosymbiosis between the ciliate *Paramecium bursaria* and *Chlorella* spp. is a mutualistic relationship (Kodama and Fujishima 2010; Reisser 1986). The host paramecia supply the symbiotic algae with nitrogen components and CO_2 (Albers et al. 1982; Albers and Wiessner 1985; Reisser 1976a; Reisser 1976b). In addition, the symbiotic algae are protected from infection by the *Chlorella* virus (Kawakami and Kawakami 1978; Reisser et al. 1988; Van Etten et al. 1991; Yamada et al. 2006), and the algal carbon fixation is enhanced (Kamako and Imamura 2006; Kato and Imamura 2009) inside the host cells. In return, the algae supply the host with photosynthetic products, particularly maltose and oxygen (Brown and Nielsen 1974; Reisser 1976a; Reisser 1976b; Reisser 1980; Reisser 1986).

Despite this mutually beneficial relationship, both *P. bursaria* and the symbiotic algae have retained the ability to grow without a partner. However, endosymbiosis is easily induced by mixing alga-free *P. bursaria* cells with isolated symbiotic algae (Karakashian 1975; Kodama and Fujishima 2005; Siegel and Karakashian 1959). Therefore, the symbiotic association between these species provides an excellent model for studying cell-to-cell interactions and the evolution of eukaryotic cells through secondary endosymbiosis.

Details of the algal infection process have recently been elucidated (Kodama 2013; Kodama and Fujishima 2005; Kodama and Fujishima 2007; Kodama and Fujishima 2009a; Kodama and Fujishima 2009b; Kodama and Fujishima 2010; Kodama and Fujishima 2011; Kodama and Fujishima 2012a; Kodama and Fujishima 2012b). Shortly after being mixed with alga-free *P. bursaria* cells, one or several green algae pass through the host cytopharynx and are pinched off and the vacuole comes into digestive vacuole (DV)-I. Acidified and condensed DV-II appears 0.5–1 min

after mixing as a result of acidosomal fusion, with a concurrent reduction in the intravacuolar pH to 2.4–3.0. Swollen DV-III appears at 2–3 min as a result of lysosomal fusion, which is accompanied by an increase in the intravacuolar pH to ≥6.5, and partially digested yellow algae also appear in DV-III. Condensed DV-IV appears at 20–30 min, at which stage the digested algae are become brown in color and have an extremely small diameter because of their digestion. A single green *Chlorella* sp. (SGC) appears as a result of the budding of the DV-IV membrane at 30 min. Then, SGCs start to localize beneath the host cell cortex. The localized alga finally starts to undergo cell division, after which it can establish endosymbiosis with the alga-free cells.

During the algal infection process, the first hurdle for the algae is the acquisition of resistance to the host's lysosomal enzymes in DV-III (Kodama and Fujishima 2005). It is known that the majority of algae are hardly digested if ingested into a large DV (Karakashian 1975; Kodama et al. 2007). Karakashian and Karakashian (1973) found that the digestion of dead boiled algae is delayed when they are enclosed in the same DV as live algae, which is a clear indication that live algae can influence the host's digestive processes. This influence may be due to the prevention or delay of acidification and lysosomal fusion with the DVs. Recently, we found that when symbiotic algae isolated from algae-bearing paramecia are maintained under constant-dark (DD) conditions for 24 h before being mixed with alga-free paramecia, almost all of the algae are digested in the host DVs (Kodama and Fujishima 2014). This finding suggests that some unknown factor produced in response to light is a prerequisite for algal resistance to the host's lysosomal enzymes (Kodama and Fujishima 2014). However, the exact stage of the host DV differentiation or algal infection that influences this was not clear because the detailed processes were unknown at that time. Therefore, in this study, we aimed to clarify the effects of live algae on the DV differentiation process or the algal infection process using the following methods: 1) lysosomal fusion with the DVs was detected using Gomori's staining method (Gomori 1952); 2) the pH inside the DVs was determined using LysoSensor Yellow/Blue DND-160 (LysoSensor); 3) to manipulate DV size, we used different concentrations of living symbiotic algae; and 4) to test the sensitivity of the process to the contents within the DV, we added different types of inocula, i.e., latex beads, boiled symbiotic algae, and algal cells grown under different light environments to the alga-free *P. bursaria* cells.

2 Materials and methods

2.1 Strains and cultures

The alga-free *P. bursaria* strain Yad1w was produced from cells of the *Chlorella* sp.-bearing *P. bursaria* strain Yad1g, as described in our previous papers (Kodama and Fujishima 2009a; Kodama and Fujishima 2009b). The Yad1g1N strain was produced by infecting cloned symbiotic *Chlorella variabilis* strain 1 N cells with the Yad1w cells (Kodama and Fujishima 2011). Symbiotic algae were isolated from the Yad1g1N cells. The culture medium was red pea (*Pisum sativum*) extract culture medium (Tsukii et al. 1995) in modified Dryl's solution (Dryl 1959) (KH_2PO_4 was used instead of $NaH_2PO_4{\cdot}2H_2O$), which was inoculated with a non-pathogenic strain of *Klebsiella pneumoniae* 1 day before use (Fujishima et al. 1990). In ordinary cultures, several hundred cells were inoculated into 2 ml of culture medium, and then 2 ml of fresh culture medium were added on each of the next 12 days. One day after the final feeding, the cultures were in the early stationary phase of growth, and all cells used in this study were of this phase. Cultivation was performed at 25 ± 1 °C. Algae-bearing cells were cultured under fluorescent lighting (20–30 μmol photons m^{-2} s^{-1}) using an incandescent lamp. The *Paramecium* strains used in this study were provided by Yamaguchi University, Japan, with support in part from the National Bio-Resource Project of the Japan Agency for Medical Research and Development.

2.2 Pulse labeling and chasing with symbiotic algae

Symbiotic algae were isolated from algae-bearing *P. bursaria* using previously described methods (Kodama and Fujishima 2005; Kodama et al. 2007). To evaluate the relationship between DV diameter and acidification of the DV, we needed to control the number of ingested algae. It has previously been reported that the algal reinfection ratio is roughly proportional to the algal concentration and the exposure time of alga-free *P. bursaria* cells to the algae (Weis and Ayala 1979). Furthermore, only small DVs were formed when a small quantity of algae was added to the alga-free *P. bursaria* (Kodama and Fujishima, unpubl. Data). Consequently, we adjusted the ratio of alga-free *P. bursaria* cells relative to the algae in a stepwise manner (alga-free cells:algae =1:10, 1:100, 1:1000, 1:10,000). Cell density was adjusted using a hemocytometer. In some experiments, the isolated algae were incubated under constant-light (LL) or DD conditions for 24 h at 25 ± 1 °C, as outlined previously (Kodama and Fujishima 2014). In addition, some algae were boiled for 10 min, as described previously (Kodama and Fujishima 2005). The alga-free *P. bursaria* cells were mixed with the treated symbiotic algae at densities of 5000 paramecia/ml and 5×10^4–5×10^7 algae/ml for 1.5 min, washed, and chased, as outlined previously (Kodama and Fujishima 2005; Kodama and Fujishima 2007; Kodama et al. 2007). In this study, the algal reinfection ratio was defined as the ratio of *P. bursaria* cells containing SGCs beneath the host cell cortex 24 h after algal mixing, as outlined in our previous study (Kodama and Fujishima 2005). The cells were observed under a

differential-interference contrast (DIC) and fluorescence microscope (BX51; Olympus Corp.). Part of the cell image was captured digitally using an Olympus DP73 camera system (Olympus Corp.). Within 15 min after mixing with the algae, the diameter of the alga(e)-containing DVs was measured using ImageJ software (NIH).

2.3 Gomori's staining of the pulse-labeled *P. bursaria* cells

To detect lysosomal acid phosphatase (AcPase) activity, a 500-μl aliquot of the cell suspension was fixed 3 min after mixing it with the algae by adding an equal volume of 4.0 % glutaraldehyde in 0.2 M cacodylate buffer containing 16.0 % sucrose (pH 7.2) for 30 min at 4 °C. The fixed cells were washed three times with the cacodylate buffer, after which the cells were incubated in 1 ml of Gomori's staining solution (Gomori 1952). AcPase activity was detected under a DIC microscope, as described previously (Kodama and Fujishima 2008; Kodama and Fujishima 2009b). AcPase activity indicated by a black precipitate in DVs demonstrates that lysosomal fusion to the DVs has occurred. In this study, DVs were classified into three different types according to the localization of their AcPase activity, based on our previous study (Kodama and Fujishima 2009b), as follows: AcPase-negative DVs (Fig. 1a-D and d-D, arrow); DVs with a partial AcPase-positive area near the DV membrane (Fig. 1b-D and e-D); and entirely AcPase-positive black DVs (Fig. 1c-D and f-D).

2.4 LysoSensor yellow/blue DND-160 staining of the pulse-labeled *P. bursaria* cells

To measure the pH inside the DVs, a 500-μl aliquot of the cell suspension was stained with LysoSensor Yellow/Blue DND-160 (LysoSensor, Molecular Probes Inc.) for 10 min under DD conditions at various times after mixing with algae, as described in our previous study (Kodama and Fujishima 2013). Within 15 min after mixing, the cells were observed under a fluorescence microscope (BX51; Olympus Corp.) to determine the pH inside the DVs.

2.5 Pulse labeling and chasing with latex spheres

The alga-free *P. bursaria* cells were mixed with polystyrene latex spheres (Difco; diameter, 3.00 μm) at densities of 5000 paramecia/ml and 5×10^7 latex spheres/ml for 1.5 min, washed, and chased, as outlined previously (Kodama and Fujishima 2005; Kodama and Fujishima 2007; Kodama et al. 2007). The cells were stained using LysoSensor for 10 min under the DD condition and observed under a fluorescence microscope as shown above.

3 Results and discussion

3.1 AcPase activity of DVs containing live or boiled algae

Three minutes after mixing with live or boiled algae, all three types of DVs were observed in alga-free *Paramecium* cells (Fig. 1). When live algae were ingested by the *Paramecium* cells, few AcPase-positive DVs were observed, whereas when boiled algae were added to the *Paramecium* cells, few AcPase-negative DVs were observed, with nearly all DVs exhibiting AcPase activity. The percentage of each type of DV that was detected 3 min after mixing with live or boiled algae is shown in Fig. 2. When live algae were ingested, approximately 70 % of DVs were AcPase-negative, as shown in Fig. 1a-D, and approximately 10 % of DVs displayed AcPase activity, as shown in Fig. 1c-D. By contrast, when boiled algae were ingested, the percentage of AcPase-negative DVs was approximately 25 %, with nearly all DVs exhibiting either partial AcPase activity (approximately 55 %) or full AcPase activity (approximately 25 %). Our previous study revealed that even when live algae were ingested, the majority of DVs became AcPase-positive within 30 min of mixing (Kodama and Fujishima 2009b). The results from the present study indicate that AcPase activity appears earlier in DVs that contain boiled algae than in those that contain live algae. There are two possible explanations for this finding: either lysosomal fusion occurs earlier in DVs containing boiled algae, or live algae suppress AcPase activity 3 min after mixing with the algae. We also found no correlation between the diameter of DVs and the intensity of AcPase activity (data not shown).

3.2 pH inside DVs containing live or boiled algae and latex spheres

Fok et al. (1987) demonstrated that the use of ionophores, weak bases, and cytochalasin B inhibited acidosome-DV fusion or reduced both the acidification rate and the pH of the DVs, which in turn inhibited lysosome-DV fusion (Fok et al. 1987). These results revealed that acidosomal fusion is needed for lysosomes to fuse with the DVs. Therefore, to confirm whether acidosomal fusion had occurred, the pH of DVs containing live algae, boiled algae, or latex spheres was measured before lysosomal fusion to the DVs using LysoSensor. In live cells, LysoSensor accumulates in acidic organelles or components and exhibits yellow fluorescence, whereas it displays blue fluorescence in less acidic conditions.

One hour after mixing alga-free *P. bursaria* with live algae, boiled algae, or latex spheres, the cells were stained with LysoSensor for 10 min and then observed within 10 min with no fixation (Fig. 3). When live algae were added to the alga-free *P. bursaria*, some cells formed large DVs, as shown in

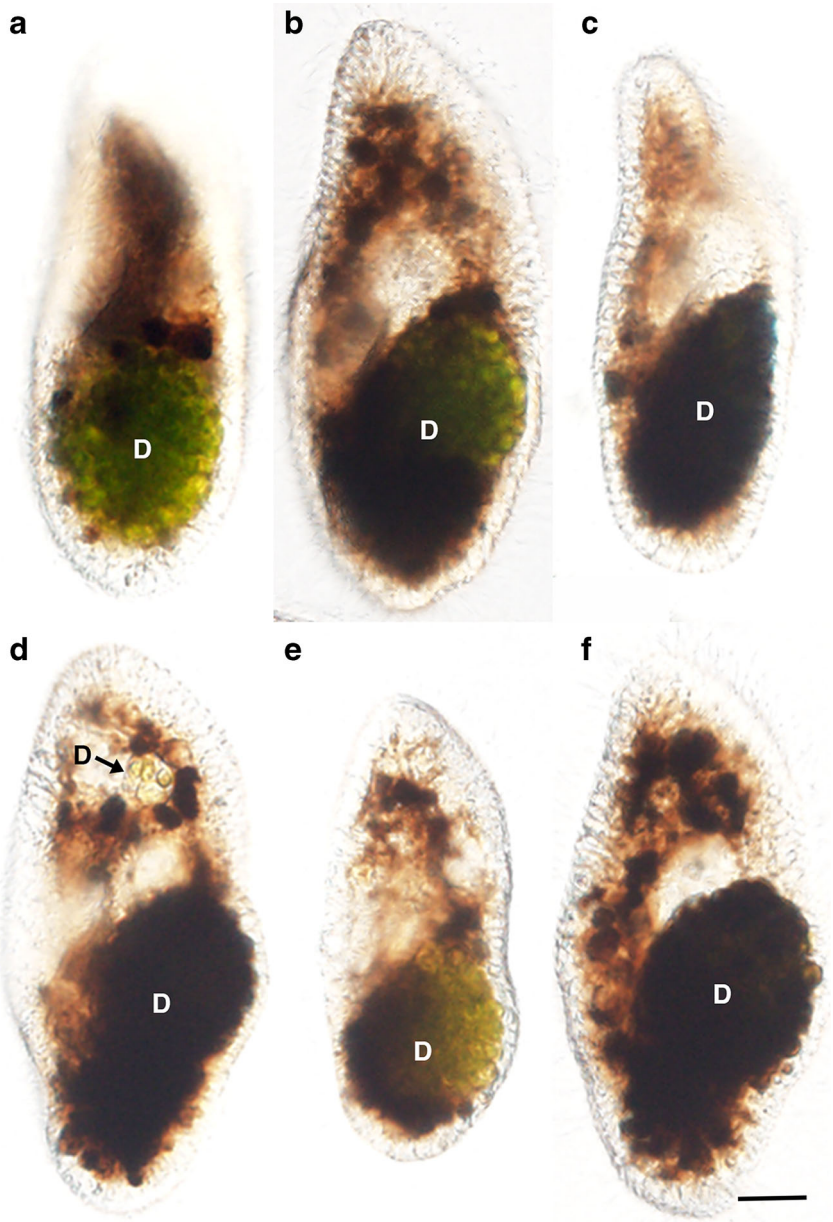

Fig. 1 Photomicrographs of alga-free *Paramecium* cells that have ingested live or boiled algae**.** Three minutes after mixing with live (**a**–**c**) or boiled (**d**–**f**) algae, acid phosphatase (AcPase) activity was detected using Gomori's staining. In both cases, all three types of digestive vacuole (DV)s classified according to the localization of their AcPase activity were observed: AcPase-negative DVs (**a**-*D* and **d**-*D*, arrow); DVs with a partial AcPase-positive area near the DV membrane (**b**-*D* and **e**-*D*); and entirely AcPase-positive black DVs (**c**-*D* and **f**-*D*). D shows a DV containing algae. The reproducibility of these results was confirmed eight times. Bar, 20 μm

Fig. 3a (arrowhead). No yellow or blue fluorescence was observed in such large DVs, as only red autofluorescence was noted in the algal chloroplast. By contrast, the small DVs (Fig. 3a, arrow) displayed yellow fluorescence. These results suggest that acidification of the small DVs, which contained only a few algae, occurred earlier than that of the large DVs, which contained more than 10 algae. When boiled algae were added to the alga-free *P. bursaria*, large DVs also exhibited yellow fluorescence (Fig. 3b, arrow). When latex spheres were added, large DVs were rarely formed for some unknown reason; however, the small DVs (Fig. 3c, arrow) displayed yellow fluorescence. Although latex spheres are excreted without digestion, this result illustrates that acidosomal fusion to the DVs containing latex spheres occurred normally. Indeed, Karakashian (1975) previously revealed that AcPase activity appears in DVs containing bacteria or inert particles such as carmine, Celkate®, or latex spheres (Karakashian 1975).

3.3 Algal reinfection ratio at varying cell densities

As shown in Fig. 3a (arrowhead), no yellow or blue fluorescence was observed in large DVs. By contrast, the small DVs (Fig. 3a, arrow) exhibited yellow fluorescence. These observations suggest that there may be some relationship between the diameter of DVs and their pH. To evaluate the relationship between DV diameter and acidification, we added symbiotic algae in a stepwise manner to alga-free *P. bursaria*. As shown in Fig. 4, when the algae were extremely rare (alga-free

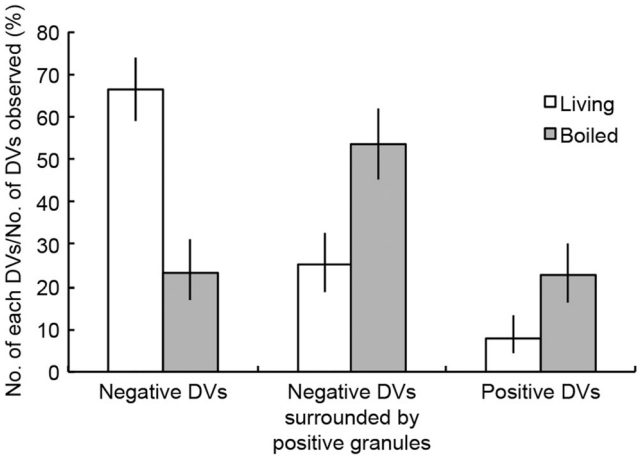

Fig. 2 Percentage of each type of DV 3 min after mixing *Paramecium bursaria* with live or boiled algae**.** Note that most of the digestive vacuole (DV)s containing live algae exhibited no acid phosphatase (AcPase) activity, as shown in Fig. 1 **a**-*D*, whereas the majority of DVs containing boiled algae displayed AcPase activity, as shown in Fig. 1 **e**-*D* and **f**-*D*. Bar, 90 % confidence limit. The reproducibility of these results was confirmed five times. For each experiment, more than 150 cells were counted. A statistically significant difference was found between live algae and boiled algae in three types of DVs ($p < 0.01$ by Fisher's exact test)

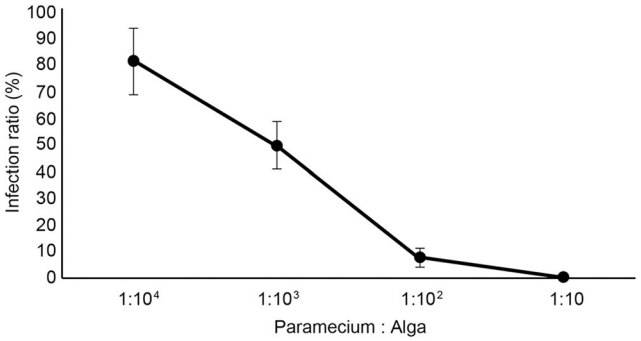

Fig. 4 The relationship between the algal reinfection ratio and the ratio of alga-free cells to algae. Alga-free *Paramecium bursaria* cells were mixed with the living algae at the densities of 5000 paramecia/ml and 5×10^4–5×10^7 algae/ml for 1.5 min, washed, and chased. The algal reinfection ratio was examined 24 h after the algal mixing by observing the percentage of cells with single green *Chlorella* sp. beneath the cell cortex. The algal reinfection ratio was roughly proportional to the concentration of alga-free *P. bursaria* cells to algae, as shown in a preliminary study (Weis and Ayala 1979). Error bars represent the standard deviation from the mean ($n = 3$). More than 500 cells were observed in each experiment

cells:algae =1:10), the algal reinfection ratio was 0 %; in addition, only alga(e)-containing DVs with extremely small diameters were formed, and some paramecia did not have any DVs (data not shown). When the algae were rare (alga-free cells:algae =1:100), the algal reinfection ratio was approximately 5 %, and alga(e)-containing DVs with both large and small diameters were observed (data not shown). By contrast, when the algae were abundant (alga-free cells:algae =1:1000 or 1:10,000), the algal reinfection ratio was approximately 50 % or 80 %, respectively, and only DVs with large diameters that contained many green algae were observed (data not shown). Consequently, we adjusted the ratio of alga-free *P. bursaria* cells and algae to a ratio of 1:100 for observation of alga(e)-containing DVs with both small and large diameters during the algal reinfection process.

3.4 Relationship between the pH and mean diameter of the DVs

As shown in Fig. 5a, some DVs exhibited yellow fluorescence (arrow), whereas others had no fluorescence (arrowhead). It should also be noted that few large DVs (see Fig. 3a, arrowhead) were observed. The mean diameters and associated fluorescent colors are shown in Fig. 5b. The mean diameter of DVs with yellow fluorescence was 5.2 ± 1.9 μm ($n = 249$),

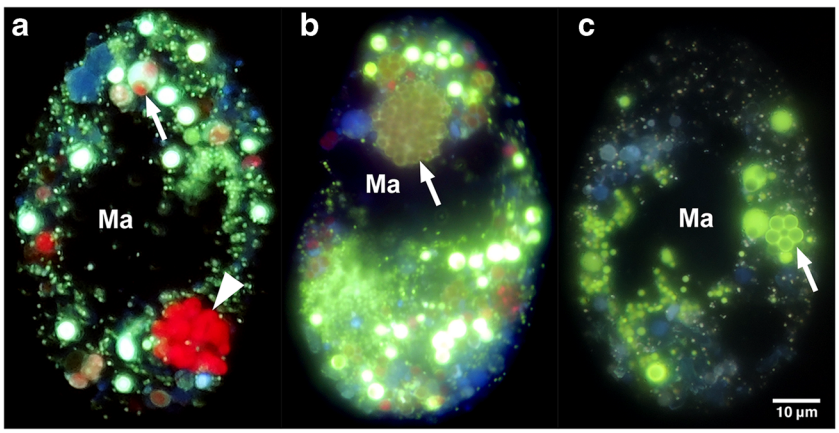

Fig. 3 Fluorescence photomicrographs of LysoSensor-treated alga-free *Paramecium bursaria* cells. Alga-free *P. bursaria* cells were mixed with living algae (**a**), boiled algae (**b**), or latex spheres (**c**) for 1.5 min. One hour after mixing, the cells were stained with LysoSensor for 10 min and observed under a fluorescence microscope. A large digestive vacuole (DV) containing many living algae exhibited no fluorescence (**a**, arrowhead), whereas a small DV containing a few algae displayed yellow fluorescence (**a**, arrow). By contrast, DVs containing boiled algae (**b**, arrow) or latex spheres (**c**, arrow) exhibited yellow fluorescence irrespective of their diameter. Ma, macronucleus. The reproducibility of these results was confirmed three times. More than 50 cells and 200 DVs were observed in each experiment

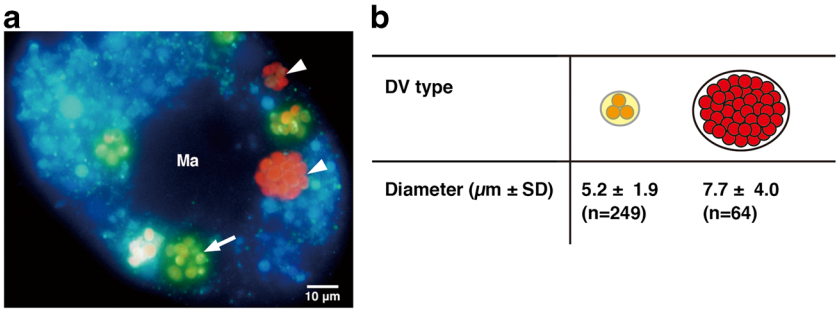

Fig. 5 Relationship between the pH inside the digestive vacuole (DV)s and their diameter. **a** Fluorescence was observed in DVs when alga-free *Paramecium bursaria* cells (5000 cells/ml) were added to 5×10^5-cells/ml living algae. Some DVs displayed no fluorescence (arrowhead), whereas others exhibited yellow fluorescence (arrow). **b** The relationship between the fluorescent colors inside DVs and their mean diameter. Note that small DVs displayed yellow fluorescence, whereas large DVs exhibited no fluorescence. Ma, macronucleus. The reproducibility of these results was confirmed three times

whereas that of DVs with no fluorescence was 7.7 ± 4.0 μm (n = 64). In a previous study, we observed that all DVs containing living or boiled algae exhibit AcPase activity 30 min after mixing with each alga and with alga-free *P. bursaria* cells (Kodama and Fujishima 2009b). Accordingly, this result reveals that lysosomes fused to all DVs within 30 min. Fok et al. (1987) examined whether the acidification of the DVs is required for lysosome-DV(s) fusion in *Paramecium* as noted previously, and they concluded that inhibition of acidosome-DV fusion or a reduction in both the acidification rate and pH of the DVs would inhibit lysosome-DV(s) fusion. Taken together, these results suggest that all DVs underwent acidification during the digestive process. Our results indicate that acidification tends to occur earlier in small DVs than in larger DVs. Because acidification of the small DVs was observed even when living algae were ingested, it can also be argued that it is not simply that the acidification of DVs containing boiled algae occurred earlier than that of DVs containing living algae. Therefore, it is possible that the living algae may secrete some inhibitor of DV acidification when large numbers of algae are ingested by one DV membrane.

3.5 pH inside DVs containing LL- or DD-incubated algae

How can the living algae influence acidification of the host's DVs? Our previous study suggested that symbiotic algae that are incubated under DD conditions for 24 h lose the ability to avoid digestion by the host's lysosomal enzymes inside the DVs (Kodama and Fujishima 2014). Therefore, in the present study, the isolated symbiotic algae were incubated under LL or DD conditions and then mixed with alga-free *P. bursaria* cells. Immediately after algal mixing, fluorescence of the LysoSensor was observed in the DVs containing LL- or DD-incubated algae (Fig. 6). Because the ratio of alga-free *P. bursaria* cells and LL- or DD-incubated algae in this experiment was 1:100, the diameter of the most of the DVs containing LL- or DD-incubated algae were small and they showed yellow fluorescence as shown by Fig. 6, arrows. When LL-incubated algae were ingested, we observed more than 200 DVs and about 80 % of them showed yellow fluorescence (arrow, Fig. 6). About 20 % of the DVs showed no fluorescence (arrowhead, Fig. 6a). However, when DD-incubated algae were ingested, we observed more than 200 DVs and all of them displayed yellow fluorescence (arrow, Fig. 6b). This result suggests that some unknown factor that is produced in response to light may be influence acidification of the host's DVs.

3.6 Summary

The results from this study and our previous studies are summarized in Fig. 7. We found that when boiled algae or latex spheres were ingested by alga-free *P. bursaria* cells, acidification of the DVs occurred irrespective of their diameter. As

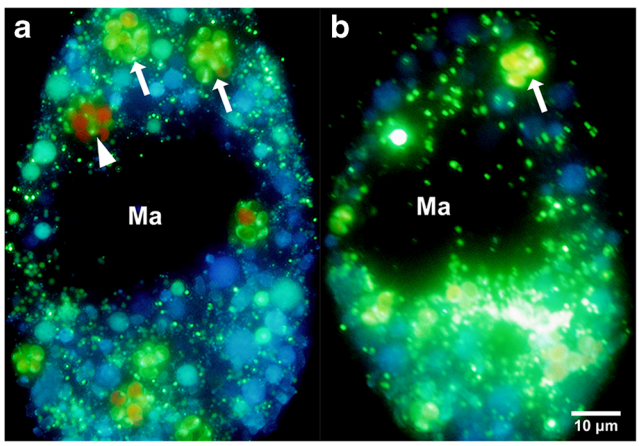

Fig. 6 Fluorescence of the digestive vacuole (DV)s of alga-free *Paramecium bursaria* cells after mixing with algae that were incubated under constant-light (LL) (**a**) or constant-dark (DD) (**b**) conditions for 24 h. As shown in (**a**), red (no LysoSensor fluorescence, arrowhead) or yellow (arrow) fluorescence was observed inside the DVs when the alga-free *P. bursaria* cells ingested LL-incubated algae. By contrast, all of the DVs exhibited yellow fluorescence (arrow) when the alga-free *P. bursaria* cells ingested DD-incubated algae. Ma, macronucleus. The reproducibility of this result was confirmed twice. For each experiment, more than 50 cells and 200 DVs were observed

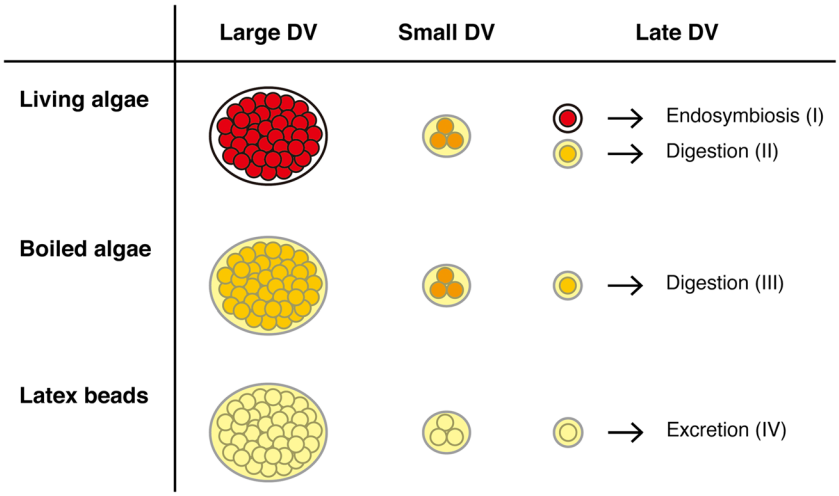

Fig. 7 Summary of the findings of this study and our previous studies. When living algae, boiled algae, and latex spheres were added to the alga-free *Paramecium bursaria* cells, digestive vacuole (DV)s of varying diameter were formed. Only DVs that contained many living algae exhibited delayed acidification. Note that the vacuoles containing one living alga that were observed 30 min after mixing with alga-free *P. bursaria* cells (late DV) displayed no yellow fluorescence (I)

explained in the Introduction, the appearance of SGCs from the DV membrane is an indispensable step in the establishment of endosymbiosis between alga-free *P. bursaria* and isolated living symbiotic algal cells. To date, we have clarified that budding from the DV membranes is also induced by boiled algae or 3.00-μm-diameter latex spheres (Kodama and Fujishima 2005; Kodama and Fujishima 2009a; Kodama and Fujishima 2009b; Kodama and Fujishima 2012b). Late DVs that contained single boiled or single latex spheres were observed 30 min after mixing with alga-free *P. bursaria* cells (Kodama and Fujishima 2005; Kodama and Fujishima 2012b). Boiled algae inside DV are digested, and their undigested cell walls are excreted by the host cytoproct. Conversely, latex spheres inside DV are excreted without digestion (data not shown). Such DVs showed yellow fluorescence (Fig. 7III and IV). By contrast, when living algae were ingested, acidification occurred inside the DVs, and this occurred earlier inside small DVs than large DVs. DVs containing both SGCs and single digested *Chlorella* sp. (SDC)s were observed 30 min after mixing living algae with alga-free *P. bursaria* cells, as shown in our previous study (Kodama and Fujishima 2005). Yellow fluorescence was only observed inside DVs containing SDCs (Fig. 7II); however, DVs containing SGCs that were localized beneath the host cell cortex only exhibited algal chloroplastic red fluorescence, as shown in Fig. 8, arrowhead and Fig. 7I. The perialgal vacuole (PV) membrane differentiates from the DV membrane soon after the alga appears from the DV membrane (Kodama and Fujishima 2009b). The PV membrane differs from the DV membrane in the following manners: (1) the PV membrane encloses only a single alga (Gu et al. 2002; Karakashian and Rudzinska 1981); (2) the gap separating the algal cell wall and the PV membrane is approximately 0.05 μm, and thus, the PV membrane can be observed using a transmission electron microscope (Reisser 1986); (3) the PV diameter does not vary greatly (2.5–4.5 μm), except during algal cell division (Reisser 1992); (4) the PV does not participate in cyclosis, but rather, it localizes beneath the host cell cortex (Kodama and Fujishima 2005; Reisser 1986); and (5) the density and distribution of particles in the PV membrane do not indicate any endocytotic or exocytotic activity, which can be observed in the DV membrane (Meier et al. 1984). Because LysoSensor fluorescence was only observed inside the DV membrane (Fig. 8, arrow) and not inside the PV membrane (Fig. 8 arrowhead), the timing of the disappearance of this fluorescence should be considered an indicator of the timing of differentiation of the PV membrane from the DV membrane. The

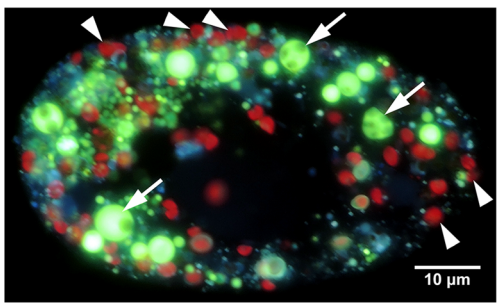

Fig. 8 Fluorescence photomicrographs of LysoSensor-stained alga-free *Paramecium bursaria* 2.5 h after mixing with algae. Alga-free *P. bursaria* cells and symbiotic algae were mixed, washed, and observed 2.5 h after mixing by staining with LysoSensor. Arrowhead shows single green alga localized beneath the host cell cortex. These algae started to increase in number via cell division and established endosymbiosis. Yellow fluorescence was observed only in the digestive vacuoles (arrows) and was not observed in the perialgal vacuoles (arrowhead). The reproducibility of this result was confirmed more than 5 times. For each experiment, more than 20 cells were observed

findings shown in Fig. 6 indicate that it is possible that some photosynthetic product(s) produced by the algae may be involved in the alteration of the DV differentiation process. Further study is required to identify the exact nature of the material that controls the differentiation of the host's DV.

4 Conclusions

We examined whether the symbiotic *Chlorella* sp. of the ciliate *Paramecium bursaria* can influence the host digestion process. Acidification and lysosomal fusion occurred later in DVs that contained living algae than in those containing boiled algae or latex spheres. These results suggest that some unknown factor in the algae that is produced in response to light may be associated with alteration of the host digestive process and indicate that the living algae can influence the host digestive processes during the early stage of algal infection.

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Compliance with ethical standards

Conflict of interest Yuuki Kodama, Miyuki Nagase and Akane Takahama declare that they have no conflict of interest.

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