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Infectivity of *Chlorella* species for the ciliate *Paramecium bursaria* is not based on sugar residues of their cell wall components, but on their ability to localize beneath the host cell membrane after escaping from the host digestive vacuole in the early infection process

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Summary. *Paramecium bursaria* cells harbor several hundred symbiotic algae in their cytoplasm. Algae-free cells can be reinfected with algae isolated from algae-bearing cells or cultivated *Chlorella* species through the digestive vacuoles. To determine the relationship between the infectivity of various *Chlorella* species and the nature of their cell wall components, algae-free *P. bursaria* cells were mixed with 15 strains of cultivated *Chlorella* species and observed for the establishment of endosymbiosis at 1 h and 3 weeks after mixing. Only 2 free-living algal strains, *C. sorokiniana* C-212 and *C. kessleri* C-531, were maintained in the host cells, whereas free-living *C. sorokiniana* C-43, *C. kessleri* C-208, *C. vulgaris* C-27, *C. ellipsoidea* C-87 and C-542, *C. saccharophila* C-183 and C-169, *C. fusca* var. *vacuolata* C-104 and C-28, *C. zofingiensis* C-111, and *C. protothecoides* C-150 and C-206 and the cultivated symbiotic *Chlorella* sp. strain C-201 derived from *Spongilla fluviatilis* could not be maintained. These infection-incapable strains could escape from the host digestive vacuole but failed to localize beneath the host cell membrane and were eventually digested. Labeling of their cell walls with Alexa Fluor 488-conjugated wheat germ agglutinin, GS-II, or concanavalin A, with or without pretreatment with 0.4 N NaOH, showed no relationship between their infectivity and the stainability with these lectins. Our results indicate that the infectivity of *Chlorella* species for *P. bursaria* is not based on the sugar residues on their cell wall and on the alkali-insoluble part of the cell wall components, but on their ability to localize just beneath the host cell membrane after escaping from the host digestive vacuole.

Keywords: *Paramecium bursaria*; *Chlorella* sp.; Concanavalin A; Wheat germ agglutinin; Lectin GS-II; Infection; Endosymbiosis.

Abbreviations: Con A concanavalin A; DIC differential-interference contrast; DV digestive vacuole; PV perialgal vacuole; WGA wheat germ agglutinin.

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Introduction

Cells of the ciliate *Paramecium bursaria* harbor several hundreds of symbiotic algae in their cytoplasm. Each symbiotic alga is enclosed in a perialgal vacuole (PV) derived from the host digestive vacuole (DV), which protects the alga from lysosomal fusion (Karakashian and Rudzinska 1981, Gu et al. 2002). Algae-free *P. bursaria* can be easily produced from algae-bearing cells by several methods: rapid fission (Jennings 1938), cultivation in darkness (Karakashian 1963, Pado 1965, Weis 1969), X-ray irradiation (Wichterman 1948), treatment with 3-(3,4-dichlorophenyl)-1,1-dimethylurea (DCMU), a blocker of electron flow in photosystem II (Reisser 1976), and treatment with the herbicide paraquat (Hosoya et al. 1995). When algae-free *P. bursaria* are mixed with symbiotic algae isolated from the symbiotic *P. bursaria*, most of the algae ingested into the host DVs are digested, but some escape and succeed in living in the host cytoplasm (Karakashian 1975). In contrast to a previously published study (Meier and Wiessner 1989), we reported that an alga can establish endosymbiosis by escaping from the host DVs after acidosomal and lysosomal fusion with the vacuole and attaching quickly to the cell membrane of the host (Kodama and Fujishima 2005).

Karakashian and Karakashian (1965) and Bomford (1965) reported the successful infection of algae-free *P. bursaria* with several strains of free-living algae. However, symbiotic *Chlorella* species derived from *Stentor polymorphus* or *Spongilla fluviatilis* were digested (Bomford

1965). Weis (1980), Reisser et al. (1982), and Nishihara et al. (1996) reported that the infection ratio of algae-free cells decreased if the algae were pretreated with concanavalin A (Con A). However, it is unclear which of the following phenomena was inhibited by Con A, namely: algal escape from the host DVs, algal localization beneath the host cell membrane, algal protection from host lysosomal fusion, or algal cell division. Takeda et al. (1998) reported that "infection-capable" *Chlorella* species, including symbiotic ones, can be distinguished by the presence of glucosamine as a chemical component in their rigid walls (alkali-insoluble part of the cell wall), whereas the rigid walls of "infection-incapable" species contained glucose and mannose. They suggested that the presence of glucosamine in the rigid wall of the alga seems to be a prerequisite in determining the symbiotic association between *P. bursaria* and *Chlorella* species.

This study therefore aimed to confirm the relationship between the nature of the cell wall and rigid wall of various *Chlorella* species and their infectivity. The binding reactivities of Alexa Fluor 488-conjugated lectins, namely, wheat germ agglutinin (WGA), a lectin derived from *Griffonia simplicifolia* (GS-II), and Con A, to various infection-capable and -incapable strains of *Chlorella* species were examined. Furthermore, the effects of the lectins on infectivity were also examined.

Material and methods

Cells and culture conditions

The *Chlorella* sp.-bearing *P. bursaria* strains OS1g (syngen 1, mating type IV), Dd1g (syngen 1, mating type II), KM2g (syngen unknown), and Bwk-16(C^+) (syngen 1, mating type II), and the algae-free strain OS1w produced from OS1g by growing them in dark conditions were used in this study. All strains except Bwk-16(C^+) were obtained from Dr. Isoji Miwa, Ibaraki University, Japan. Strain Bwk-16(C^+) was obtained from Dr. Toshikazu Kosaka, Hiroshima University, Japan. Furthermore, a strain OS1g1N cell, which was produced by infecting a strain OS1w cell with cloned symbiotic *Chlorella vulgaris* 1N cells, was also used (Kodama et al. 2007). The culture medium used was 1.25% fresh lettuce juice in modified Dryl's solution (MDS) (Dryl 1959) (KH_2PO_4 was used instead of $Na_2H_2PO_4{\cdot}2H_2O$), inoculated with a non-pathogenic strain of *Klebsiella pneumoniae* 1 day before use (Hiwatashi 1968). In ordinary cultures, several hundred cells were inoculated into 2 ml of culture medium and then 4, 4, 6, 6, and 6 ml of fresh culture medium were successively added every day for 5 days (Kodama and Fujishima 2005). One or two days after the final feeding, the cultures reached the early stationary phase of growth. All cells used in the present experiments were at this phase.

Fifteen strains of cultivated chlorellaceous cells – *C. vulgaris* C-27, *C. sorokiniana* C-212 and C-43, *C. kessleri* C-208 and C-531, *C. ellipsoidea* C-87 and C-542, *C. saccharophila* C-183 and C-169, *C. fusca* var. *vacuolata* C-104 and C-28, *C. zofingiensis* C-111, *C. protothecoides* C-150 and C-206, and the symbiotic *Chlorella* sp. strain C-201 derived from *Spongilla fluviatilis* – were obtained from the Institute of Applied Microbiology Culture Collection at the University of Tokyo. These algae were cultured in 0.1% Hyponex (Hyponex, Osaka, Japan) solution. To harvest the cells, the algal cultures were centrifuged at 4500 **g** for 1 min, washed twice with phosphate-buffered saline (PBS) (137 mM NaCl, 2.68 mM KCl, 8.1 mM $NaHPO_4{\cdot}12H_2O$, 1.47 mM KH_2PO_4, pH 7.2), MDS, or distilled water.

Isolation of Chlorella sp.

Symbiotic *Chlorella* sp. cells were isolated from paramecium strains OS1g, Dd1g, KM2g, Bwk-16(C^+), and OS1g1N. Cells from a 600 ml culture of each strain at the early stationary phase of growth were strained through 8 layers of fine gauze to remove gross debris. The cells were harvested by centrifugation at 300 **g** for 2 min at room temperature, washed twice with MDS, and centrifuged again at the same velocity and temperature. The sediment cells were suspended in 1 ml of MDS containing 0.1 mM phenylmethylsulfonyl fluoride (Sigma) and homogenized by hand in a Teflon homogenizer on ice. Following Takeda et al. (1998), the homogenate was transferred to a plastic centrifuge tube (volume, 50 ml) that had been sliced at the bottom and equipped with a 15 μm pore size nylon mesh (Kyosin Rikoh, Tokyo, Japan). Immediately, 5 ml of MDS was added to the tube, and the algae in the filtrate were harvested and washed twice with 10 ml of MDS or PBS by centrifugation at 4500 **g** for 1 min at 4 °C. The density of the chlorellaceous cells was counted with a blood-counting chamber.

Treatment of symbiotic and free-living Chlorella species with lectins

Alexa Fluor 488-conjugated WGA, GS-II, and Con A (Molecular Probes) were dissolved in PBS containing 2 mM sodium azide. GS-II was dissolved in PBS containing 1 mM $CaCl_2$. These stock solutions were stored at −80 °C until use. These stock solutions were diluted with PBS without sodium azide to give final concentrations of 100, 200, and 2000 μg of each lectin per ml. In the case of GS-II, the stock solution was diluted with PBS containing 1 mM $CaCl_2$. These diluted solutions were stored at 4 °C until use. For lectin treatments, 20 μl aliquots of algal suspension (10^8 cells per ml) in MDS and an equal volume of lectin solution at a concentration of 100 μg/ml were incubated in the dark for 2 or 3 h. After incubation, they were washed twice with PBS, centrifuged at 4500 **g** for 1 min at 25 °C, and observed with a fluorescence microscope (Olympus, BX60). More than 100 algae were observed and the ratio of cells with fluorescence was determined. In a separate procedure, prior to lectin treatments, the same amount of algal suspension aliquots were pretreated with 1 ml of 0.4 N NaOH, incubated at 37 °C for 20 h as described by Takeda and Hirokawa (1978), and washed twice with distilled water by centrifuging at 4500 **g** for 1 min. To test the possibility that the isolation of the algae from the host cells may affect the algal cell wall, algae were pushed out from the host cell onto a glass slide, air dried, and treated with lectins as described above. A different procedure for Con A treatment was also performed. Prior to treatment, algae isolated from strain OS1g were fixed with 2.5% (v/v) glutaraldehyde dissolved in 0.1 M phosphate buffer, pH 7.4, for 1 h.

Pulse-label and chase with isolated Chlorella spp.

Algae isolated from paramecium strain OS1g were treated with equal volumes of Con A solutions (200 and 2000 μg/ml) following the procedure described above. After incubation, they were washed 5 times with MDS by centrifuging at 4500 **g** for 1 min at 25 °C. The Con A-treated and the untreated algae (5×10^7 algae per ml) were mixed with the algae-free paramecium strain OS1w (5×10^3 paramecia per ml) in a centrifuge tube (volume, 10 ml) for 1.5 min and 24 h. The same procedure was also performed for free-living and symbiotic algae, but the duration of mixing with paramecia was 1 h. After mixing, the algae that were not engulfed by paramecia were removed through a 15 μm pore size nylon

mesh. The paramecia retained in the mesh were transferred to a centrifuge tube (volume, 10 ml) and resuspended in 1 ml MDS and then chased for various times. A 100 µl aliquot of the cell suspension was fixed with 200 µl of 4% (w/v) paraformaldehyde after various time intervals, and the cells were observed under a DIC (differential-interference contrast) and fluorescence microscope.

In the same way, *C. sorokiniana* (strain C-212) cells were treated with WGA, mixed with strain OS1w cells, and the fates of the algae were traced by the pulse-label and chase method. The paramecia used in this procedure were fed with 2 ml of culture medium every 3 days after mixing with *C. sorokiniana* cells.

Throughout the duration of this experiment, all setups were kept at 25 ± 1 °C under a fluorescent light (1500 lux), unless otherwise mentioned. The reproducibility of all results was confirmed more than twice.

Results and discussion

Infectivity of symbiotic and free-living Chlorella species for P. bursaria

Takeda et al. (1998) reported that the free-living *Chlorella* species *C. vulgaris*, *C. kessleri*, and *C. sorokiniana* could infect *Chlorella* sp.-free *P. bursaria* cells as efficiently as the symbiotic *Chlorella* sp. cells isolated from *Chlorella* sp.-bearing *P. bursaria* cells, but *C. ellipsoidea*, *C. saccharophila*, *C. luteoviridis*, *C. zofingiensis*, and *C. mirabilis* cells could not infect the paramecia. We examined the infectivity of 14 free-living strains of *Chlorella* species for algae-free *P. bursaria* OS1w cells, namely, *C. vulgaris* C-27, *C. sorokiniana* C-212 and C-43, *C. kessleri* C-208 and C-531, *C. ellipsoidea* C-87 and C-542, *C. saccharophila* C-183 and C-169, *C. fusca* var. *vacuolata* C-104 and C-28, *C. zofingiensis* C-111, and *C. protothecoides* C-150 and C-206 and the symbiotic *Chlorella* sp. strain C-201 derived from *Spongilla fluviatilis*. The infection experiments were carried out by mixing the algae (5×10^7 algae per ml) and algae-free paramecia (5×10^3 paramecia per ml) for 1 h, washing, chasing, and then fixing at 1 h and 3 weeks after mixing.

The paramecium cells were classified into 4 groups depending on the numbers of algae in the cells, i.e., 0, 1–5, 6–20, and more than 20, and the proportions of these cells are given in Fig. 1. One hour after mixing, all observed paramecia contained green algae, though the mean numbers retained in the cells differed among the algal strains used (Fig. 1A). *Chlorella ellipsoidea*, *C. saccharophila* and *C. fusca* var. *vacuolata* were not efficiently ingested com-

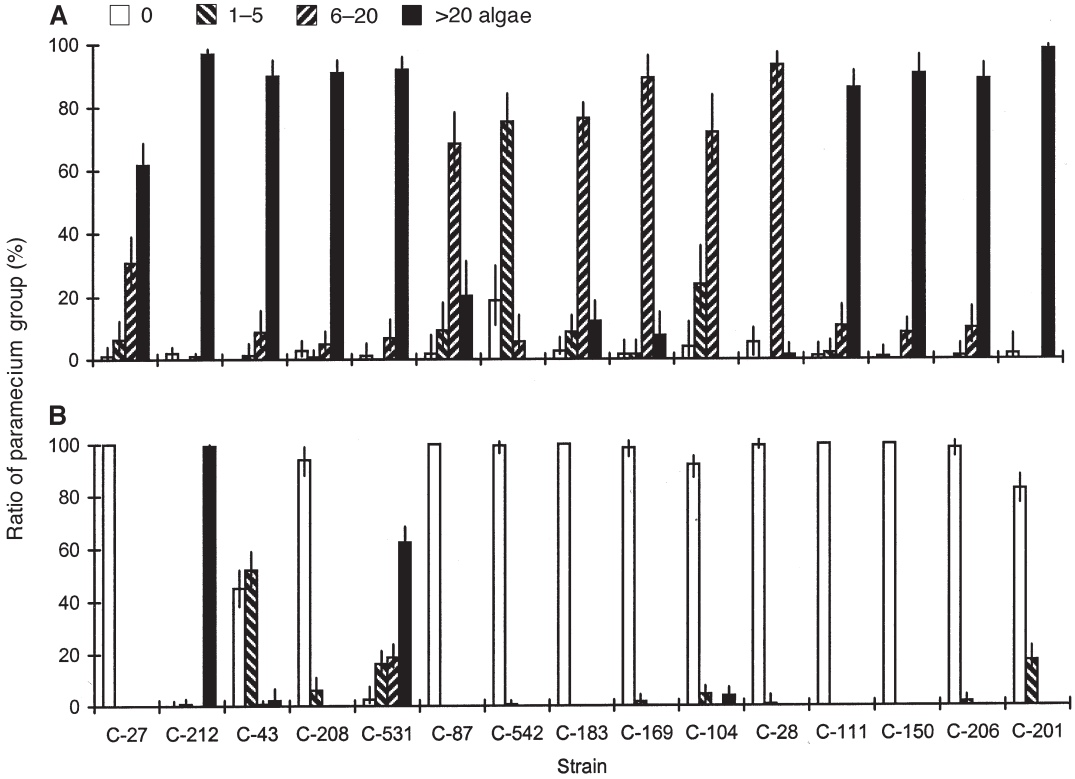

Fig. 1. Infectivity of symbiotic and free-living *Chlorella* species for *P. bursaria* 1 h (**A**) and 3 weeks (**B**) after mixing with algae-free paramecia. The paramecia were placed into 4 groups according to the number of algae ingested: 0 algae, 1–5 algae, 6–20 algae, and more than 20 algae. For both time intervals, 50–222 cells were observed. Bar in bar graph, 90% confidence limits. X-axis, strains of *Chlorella* species mixed with paramecia. Y-axis, percentage of paramecia group. Only algal strains C-212 and C-531 were maintained in the cytoplasm of the algae-free cells

pared with the other algal species. Although *C. kessleri* C-531 had a large diameter, 3.81 ± 1.03 μm (mean with standard error, n = 52), compared with that of *C. sorokiniana* C-212 (2.05 ± 0.67 μm, n = 44), both cells were efficiently ingested by *P. bursaria*. This shows that differences in the number of algae ingested by *P. bursaria* cells are not simply due to the difference in the diameter of the algae. Partially digested algae were also observed in the host DVs, as indicated by their brown color (Kodama and Fujishima 2005, Kodama et al. 2007). *Chlorella sorokiniana* C-212 and *C. kessleri* C-531 were retained in the host cells and managed to multiply by cell division. These strains could be maintained in the host cells for more than 2 years after mixing. However, *C. sorokiniana* C-43 and *C. kessleri* C-208 were not maintained for more than 4 weeks. A few cells of *C. kessleri* C-208, *C. ellipsoidea* C-542, *C. saccharophila* C-169, *C. fusca* var. *vacuolata* C-104 and C-28, *C. protothecoides* C-206, and *Chlorella* sp. strain C-201 derived from *S. fluviatilis* remained in a few paramecia for 3 weeks after mixing (Fig. 1B). However, these algae disappeared from the host cells 4 weeks after mixing. It should be noted that the infection-capable algae, *C. sorokiniana* C-212 and *C. kessleri* C-531, localized close to the host cell membrane after escaping from the host DV at 3 weeks after mixing. Dividing algal cells were also observed to localize to the same site. However, any infection-incapable algal strain examined did not localize near to the host cell membrane and did not divide. To confirm whether this phenomenon is associated with algal infectivity, an infection experiment using isolated symbiotic algae and paramecium strain OS1w was performed as described above, and the cells were fixed 3 h after mixing. In the same way, algae-free strain OS1w cells were pulse-labeled for 1.5 min with infection-incapable *C. saccharophila* C-169 cells, chased, and fixed at 3 h after mixing. It was confirmed that the isolated algae localized close to the host cell membrane after escaping from the host DV (Fig. 2A), whereas the infection-incapable algal cells did not (Fig. 2B). This suggests that algal localization beneath the host cell membrane may be a prerequisite for algal infection. It should be noted that the symbiotic algae localize at a distance of about 10 μm from each other.

The free-living *Chlorella* species examined were hardly digested in the host DVs. Host DVs containing these algae usually fused with the host cytoproct and expelled the algae outside the host cells without any distinguishable morphological digestion.

Although Takeda et al. (1998) reported that all of the aposymbiotic strains that they examined (strains T316w, Kz1w, Sj2w, Ok2w, and Uk2w) could establish endosymbiosis with all strains used (*C. vulgaris*, *C. kessleri*, and *C. sorokiniana*), the strain OS1w used in this study could only establish endosymbiosis with specific strains of *C. vulgaris*, *C. kessleri*, and *C. sorokiniana*. On the other hand, Takeda et al. (1998) reported that the free-living *C. kessleri* C-208 could infect *P. bursaria* T316w, Kz1w, Sj2w, Ok2w, and Uk2w. However, the algal strain C-208 could not infect *P. bursaria* OS1w in the present study. Thus, it appears that the establishment of endosymbiosis is both algal and host strain specific.

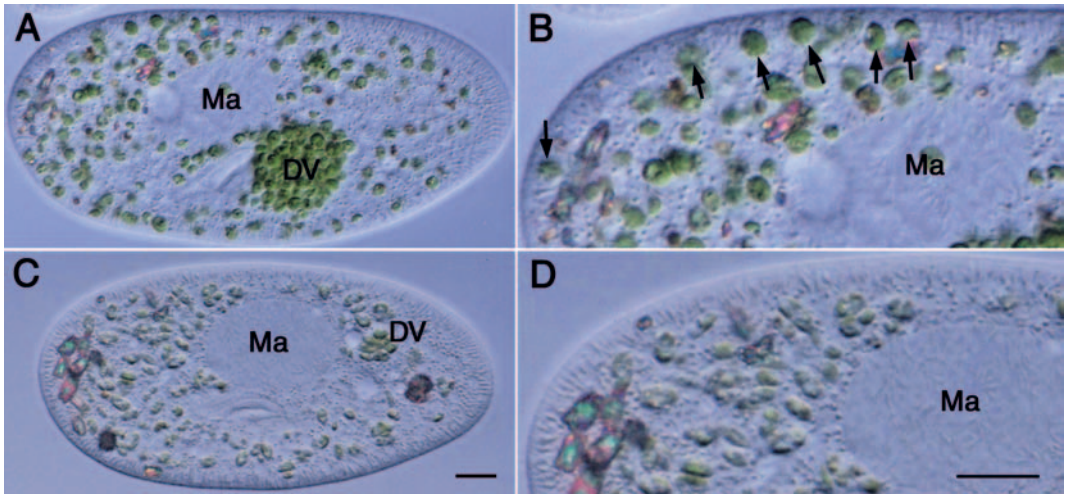

Fig. 2. Photomicrographs of *P. bursaria* OS1w cells pulse-labeled with symbiotic *Chlorella* sp. cells isolated from *P. bursaria* OS1g cells (**A** and **B**) and with *C. saccharophila* C-169 cells (**C** and **D**). Paramecia were observed 3 h after mixing. DIC. Note that the symbiotic *Chlorella* sp. cells are localized close to the host cell membrane (arrows), whereas *C. saccharophila* C-169 cells are not. *Ma* Macronucleus, *DV* digestive vacuole. Bar: 10 μm

Labeling of free-living and symbiotic Chlorella spp. by lectins

Takeda et al. (1998) showed that infection-capable species of *Chlorella* (*C. vulgaris*, *C. sorokiniana*, *C. kessleri*, and symbiotic *Chlorella* sp. obtained from *P. bursaria*) are distinguished by the presence of glucosamine in the alkali-insoluble part of their cell wall (rigid wall), whereas the rigid walls of infection-incapable species (*C. ellipsoidea*, *C. saccharophila*, *C. fusca* var. *vacuolata*, *C. zofingiensis*, and *C. protothecoides*) contain glucose and mannose. It is known that WGA binds to N-acetylglucosamine and N-acetylneuraminic acid residues (Allen et al. 1973, Goldstein et al. 1975, Yamamoto et al. 1981), GS-II binds to α- or β-linked N-acetyl-D-glucosamine (Lyer et al. 1976, Ebisu and Goldstein 1978), and Con A selectively binds to mannose residues (Poretz and Goldstein 1970, Goldstein et al. 1974, Ogata et al. 1975). In order to determine whether the infection-capable and -incapable strains of living *Chlorella* species can be distinguished by labeling with these lectins, we treated the living algae with Alexa Fluor 488-conjugated WGA, GS-II, and Con A.

After release of autospores, sporangial walls were labeled by all 3 lectins in all chlorellaceous strains examined (data not shown). However, the cell walls of nondividing algae of various chlorellaceous strains showed different lectin-binding abilities. Fluorescent labeling with WGA was observed on the cell walls of *C. sorokiniana* C-212 and C-43. *Chlorella* sp. isolated from *P. bursaria* Bwk-16(C^+) also showed fluorescence, though it was present on only 6.9% (n = 1000) of the cells. Other strains of *Chlorella* species examined did not show fluorescence. Fluorescence from GS-II was observed only on *C. saccharophila* C-183, but not on the other strains examined. Con A fluorescence was observed on *C. ellipsoidea* C-87 and C-542, *C. saccharophila* C-183 and C-169, *C. fusca* var. *vacuolata* C-104 and C-28, *C. zofingiensis* C-111, *C. protothecoides* C-150 and C-206, and strain C-201 derived from *S. fluviatilis*. *Chlorella* sp. isolated from *P. bursaria* Bwk-16(C^+) also showed fluorescence, though it was present on only 6.2% (n = 1000) of the cells. *Chlorella vulgaris* C-27, *C. sorokiniana* C-43, *C. kessleri* C-208 and C-531, and *Chlorella* sp. isolated from *P. bursaria* OS1g, Dd1g, and KM2g were not labeled by any of the lectins.

Previous studies (Weis 1980, Reisser et al. 1982, Nishihara et al. 1996) reported that symbiotic *Chlorella* sp. cells of *P. bursaria* are agglutinated by Con A if the cells are fixed with glutaraldehyde before mixing with Con A. However, despite glutaraldehyde fixation, Con A neither labeled the symbiotic *Chlorella* sp. cells (Fig. 3B, D) nor agglutinated them (Fig. 3A, C) in our experiments. Thus, our data show that there is no relationship between the infectivity of *Chlorella* species and the lectin-specific reactivity of the cell walls of living algae.

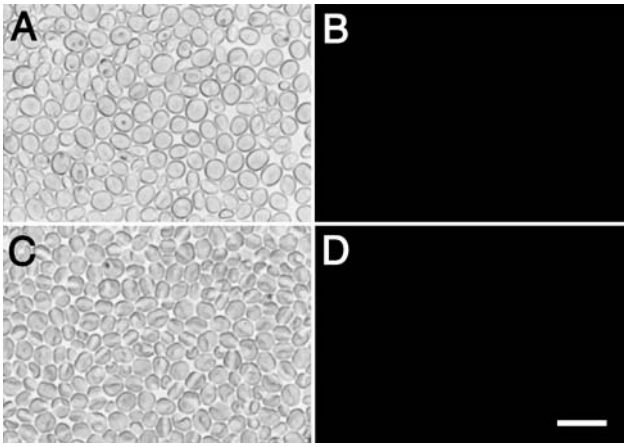

Fig. 3. Photomicrographs of Con A-treated isolated *Chlorella* sp. cells without (**A** and **B**) and with prior fixation (**C** and **D**) with glutaraldehyde. Fluorescence was not observed on cells, whether fixed (**D**) or not (**B**). Panels A and C are DIC microscopy images; panels B and D, fluorescence microscopy images. Bar: 10 μm

As reported earlier (Kodama and Fujishima 2005), boiled algae lose their ability to infect the host cell. Like living algae, boiled algae can escape from host DVs to the cytoplasm, but they fail to localize beneath the host cell membrane after escaping from the DVs and are eventually digested in the host cell. In order to test the possibility that boiling may change the algal surface components needed for localization in early infection, we compared the lectin-binding reactivities of intact and boiled algae. Algae isolated from paramecium strain OS1g were boiled for 10 min and cooled on ice. The boiled algae retained their green color, but their surface became slightly bumpy. After boiling, the algae were labeled with the 3 lectins as described above. The results revealed that none of the lectins labeled the boiled algae, thus showing that boiling did not enhance the lectin reactivity of the symbiotic algae.

To examine the lectin-binding ability of the rigid walls of various *Chlorella* species, the algae were pretreated with 0.4 N NaOH for 20 h as described by Takeda and Hirokawa (1978), treated with Alexa Fluor 488-conjugated lectins, and observed under a fluorescence microscope. More than 100 algal cells were observed in each experiment and the proportion of fluorescent cells was determined. After treatment with NaOH, the algal cells were discolored or yellow (Fig. 4C, G, K). The algae treated with NaOH were all digested in the host DV when they were added to paramecia. Compared with living algal cells, cells treated with NaOH showed different lectin-

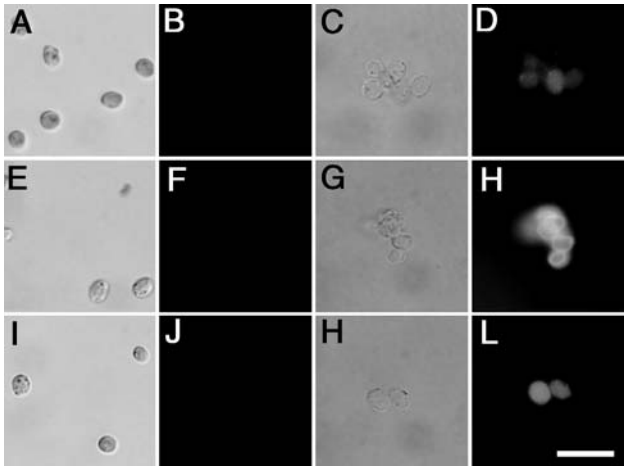

Fig. 4. Photomicrographs of *Chlorella* sp. isolated from *P. bursaria* strain OS1g treated with Con A (**A** and **B**), WGA (**E** and **F**), or GS-II (**I** and **J**). No fluorescence from the 3 lectins was observed. Panels A, E, and I are DIC microscopy images; panels B, F, and J, fluorescence microscopy images. NaOH-pretreated cells labeled with Con A (**C** and **D**), WGA (**G** and **H**), or GS-II (**K** and **L**). The lectins were able to label NaOH-pretreated cells. Panels C, G, and K are DIC microscopy images; panels D, H, and L, fluorescence microscopy. The same results were obtained for all symbiotic *Chlorella* sp. examined (Table 1). Bar: 10 μm

binding abilities (Table 1). The NaOH-treated *C. vulgaris* C-27 and *C. sorokiniana* C-43 and C-212 were labeled with WGA fluorescence, though the last strain showed only a few positive cells (2%, n = 500). Symbiotic algae obtained from *P. bursaria* OS1g, Dd1g, KM2g, and Bwk-16(C^+) also showed WGA fluorescence. GS-II fluorescence was observed on *C. vulgaris* C-27, and *C. sorokiniana* C-212 and C-43, and a few positive cells were observed for *C. kessleri* C-208 (7.4%, n = 161) and C-531 (3.4%, n = 238). Furthermore, GS-II also labeled *C. ellipsoidea* C-87 and C-542 and *C. saccharophila* C-183 and symbiotic *Chlorella* sp. strain C-201 derived from *S. fluviatilis* and those from *P. bursaria* OS1g, Dd1g, KM2g, and Bwk-16(C^+). Con A labeled *C. ellipsoidea* C-87 and C-542, *C. saccharophila* C-183 and C-169, *C. fusca* var. *vacuolata* C-104, *C. zofingiensis* C-111, *C. protothecoides* C-150 and C-206, *C. vulgaris* C-27, and *C. sorokiniana* C-43 and C-212, though only a few cells of the last strain were labeled (2%, n = 500).

The symbiotic *C. vulgaris* 1N cells also showed the same lectin-binding reactivities as the algal cells of *P. bursaria*

Table 1. Lectin-binding activity of the NaOH-treated and untreated cell walls of *Chlorella* species and their infectivity for algae-free *P. bursaria*

Species	Strain (alternative name)	Infectivity		Lectin labeling of:[a]					
		T316w	OS1w	Nontreated cells			NaOH-treated cells		
				WGA	GS-II	Con A	WGA	GS-II	Con A
C. vulgaris	C-27[b]		−	−	−	−	+	+	+
C. sorokiniana	C-212 (211-8k)[c]	+	+	+	−	−	±	+	±
	C-43[b]		−	+	−	−	+	+	+
C. kessleri	C-208 (211-11g)[c]	+	−	−	−	−	−	±	−
	C-531 (211-11h)[c]	+	+	−	−	−	−	±	−
C. ellipsoidea	C-87 (211-1a)[c]	−	−	−	−	+	−	+	+
	C-542 (211-1a)[c]	−	−	−	−	+	−	+	+
C. saccharophila	C-183[b]		−	−	+	+	−	+	+
	C-169[b]		−	−	−	+	−	−	+
C. fusca var. *vacuolata*	C-104 (211-8b)[b]		−	−	−	+	−	−	+
	C-28[b]		−	−	−	+	−	−	−
C. zofingiensis	C-111 (211-14)[b]		−	−	−	+	−	−	+
C. protothecoides	C-150 (211-11a)[b]		−	−	−	+	−	−	+
	C-206[b]		−	−	−	+	−	−	+
Symbiotic *Chlorella* sp.	C-201[b]		−	−	−	+	−	+	+
	OS1g[b]		+	−	−	−	+	+	+
	Dd1g[b]		+	−	−	−	+	+	+
	KM2g[b]		+	−	−	−	+	+	+
	Bwk-16(C^+)[b]		+	±	−	±	+	+	+
	1N[b]		+	−	−	−	+	+	+

[a] Algal cells were labeled with Alexa Fluor 488-conjugated Con A, WGA or GS-II. +, 100% of cells with FITC fluorescence; ±, less than 100%; −, 0%. For each experiment, more than 100 algal cells were observed

[b] Strain used only in this study

[c] Strain used in this study and by Takeda et al. (1998)

OS1g cells, i.e., the rigid wall was stained by WGA, GS-II, and Con A, notwithstanding that this strain is infection-capable (data not shown). Our results are inconsistent with those of Takeda et al. (1998) and clearly show that infectivity of *Chlorella* species for the ciliate *P. bursaria* is not based on the sugar residues of their cell wall components.

Con A also labeled symbiotic *Chlorella* sp. C-201 derived from *S. fluviatilis* and those from *P. bursaria* OS1g, Dd1g, KM2g, and Bwk-16(C^+). Interestingly, the 3 lectins were able to label the NaOH-treated symbiotic algae isolated from the 4 strains of *P. bursaria* but not their untreated counterparts (Fig. 4D, H, L and Table 1). It should be noted that their sporangial wall could not be detected after treatment with NaOH and was probably, therefore, lysed. Thus, our data show that there is no evident correlation between the infectivity of *Chlorella* species and the presence of glucosamine in the rigid walls, in contrast to the published study of Takeda et al. (1998).

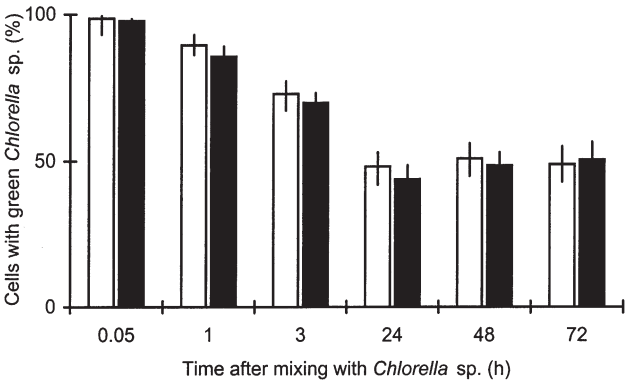

Fig. 5. Effects of low concentration (100 μg/ml) Con A during the infection process. Con A-treated isolated cells of *Chlorella* species were mixed with algae-free paramecia (see Material and methods section), washed, chased, and fixed at 0.05, 1, 3, 24, 48, and 72 h after mixing. Paramecia containing green algae were compared. For each time interval, 214–315 cells were observed. White bar, paramecia with Con A-treated algae; black bar, paramecia with untreated algae (control). Bar in bar graph, 90% confidence limits. This graph shows no significant difference between lectin-treated and untreated algae

Effect of Con A on the infectivity of symbiotic Chlorella sp.

Weis (1980), Reisser et al. (1982), and Nishihara et al. (1996) reported that the infection ratio of algae-free cells decreased if the algae were pretreated with Con A, though they did not confirm whether Con A labeled the algae or not. However, as shown in Table 1 and Figs. 3 and 4, Con A did not label symbiotic algae isolated from strain OS1g cells. To confirm the effects of Con A treatment on infectivity, isolated *Chlorella* sp. cells (5×10^8 algae per ml) were pretreated with Con A (100 μg/ml) for 3 h under constant darkness (after Weis 1980). Washed algal cells (5×10^7 algae per ml) were mixed with the OS1w cells (5×10^3 paramecia per ml) for 1.5 min, chased, and fixed at 0.05, 1, 3, 24, 48, and 72 h after mixing. The same procedure was carried out without Con A pretreatment. The results showed that almost all paramecia observed contained green algae at 0.05 h after mixing with or without Con A pretreatment (Fig. 5). This shows that both the Con A-treated and untreated algae can be ingested by the host DVs. Thereafter, the proportion of cells with green algae began to decrease, due to either digestion or excretion, and their proportion reached a plateau at about 50% at 24 h after mixing and continued up to 72 h. This further shows that about 50% of paramecium cells could establish endosymbiosis whether or not the algae were pretreated with Con A. We also performed the same experiment by mixing for 24 h with algae treated with a higher concentration of Con A (1000 μg/ml). Con A did not stain the algae (data not shown). As shown in Fig. 6, at 24 h after mixing, the higher concentration of Con A did not inhibit

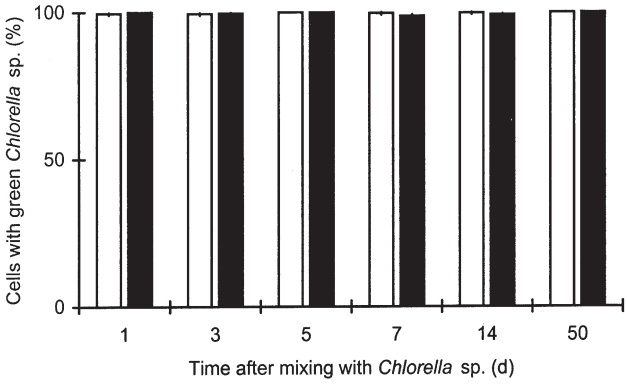

Fig. 6. Effects of high concentration (1000 μg/ml) Con A during the infection process. Isolated cells of *Chlorella* species treated with Con A were mixed with algae-free paramecia (see Material and methods section), washed, chased, and fixed at 1, 3, 5, 7, 14, and 50 days after mixing. Paramecia containing green algae were compared. For each time interval, 196–500 cells were observed. White bar, paramecia with Con A-treated algae; black bar, paramecia with untreated algae (control). Bar in bar graph, 90% confidence limits. This graph shows no significant difference between lectin-treated and untreated algae. No notable effect was observed when the Con A concentration was increased

algal infection, as observed with the lower concentration. In this experiment, the infection ratio was higher (treated, 99.6%, n = 250; untreated, 100%, n = 250). This is because many algae were ingested in the host DVs, since as the time of pulse-labeling is prolonged, the infection ratio approaches 100%, as reported by Weis and Ayala (1979). Furthermore, these *Chlorella* sp. cells were maintained in the cytoplasm of paramecia for at least 50 days after

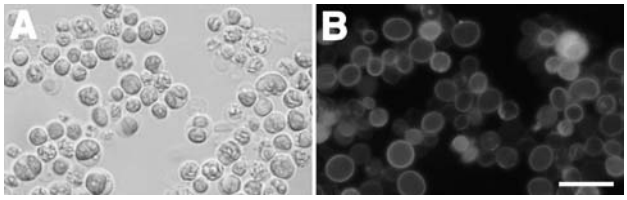

Fig. 7. Photomicrographs of *C. sorokiniana* (C-212) treated with WGA, as observed by DIC microscopy (**A**) and fluorescence microscopy (**B**). Lectin fluorescence can be observed on the cell wall surface of the algae. Bar: 10 μm

mixing. Thus, our data show that Con A does not affect the phagocytosis of paramecium cells. Moreover, no significant difference in the infection ratio was observed between the Con A-treated and the untreated *Chlorella* sp. cells.

Further investigation was also conducted to determine the Con A reactivity of algae isolated from strain OS1g cells that had been engulfed into the host DVs during the infection process. The paramecia were pulse-labeled with the algae and chased as described above, homogenized with a Teflon homogenizer by hand strokes on ice at 2 h after mixing, and observed without fixing. The results showed that the engulfed algae were not labeled by Con A. It was also confirmed that WGA and GS-II cannot label such algae (data not shown). There is a possibility that the isolation of the algae from paramecia may affect the algal cell wall. To exclude this, we used algae obtained directly from host cells ruptured on glass slides and labeled them with the three lectins. However, no fluorescence was observed (data not shown).

We observed that Con A, WGA, and GS-II labeled the paramecium cell surface, cilia, and trichocyst (data not shown). These results suggest that the observations of Weis (1980), Reisser et al. (1982), and Nishihara et al. (1996) may be due to interference resulting from phagocytosis of host cell surface membrane labeling as reported for *Dictyostelium discoideum* (Ryter and Hellio 1980) or due to strain-specific labeling of algae by Con A.

Effect of WGA on the infectivity of C. sorokiniana C-212

Chlorella sorokiniana C-212 cells are able to infect algae-free *P. bursaria* cells (Fig. 1) and can be labeled by WGA (Table 1 and Fig. 7B). To examine the effect of WGA labeling of the cell wall on infection, the algae were treated with 100 μg of WGA per ml (as described above), mixed with paramecia, chased, and fixed at 1 and 72 h after mixing. The cell surface, including cilia, of strain OS1w cells showed a faint fluorescence, probably due to WGA released from strain C-212 cells during incubation with the algae (data not shown). As shown in Fig. 8A, many algal cells were ingested by all paramecia at 1 h, and at 72 h, the proportions of paramecia containing WGA-labeled and unlabeled algae were not significantly different (Fig. 8B). In both setups, strain C-212 algae were localized very close to the cell membrane of the host and could multiply by binary fission (data not shown). Although the proportion of paramecia with more than 20 algal cells was significantly decreased when the algal cells were labeled with WGA, our data show that WGA labeling of algae does not hinder phagocytosis and does not inhibit the establishment of endosymbiosis with *P. bursaria*.

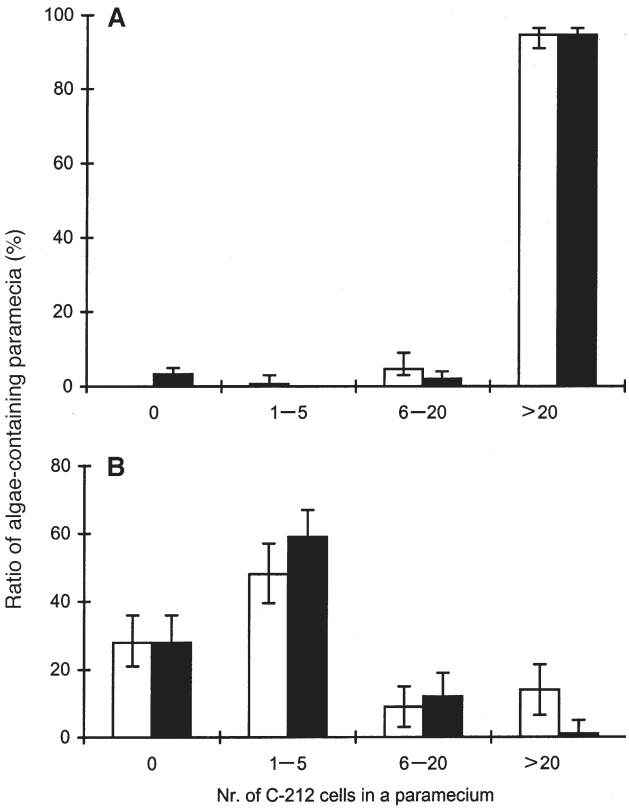

Fig. 8. Effect of WGA treatment on the infectivity of C-212 algae 1 h (**A**) and 72 h (**B**) after mixing with algae-free paramecia. For each time interval, 100–150 cells were observed. White bar, untreated algae; black bar, WGA-treated cells. Bar, 90% confidence limits. Y-axis, percentage of algae-containing paramecia. X-axis, number of C-212 algae ingested by paramecia in 4 groups (0, 1–5, 6–20, and >20 algae). Almost all paramecia ingested many C-212 algae regardless of WGA treatment

The present study therefore shows that the infectivity of infection-capable *Chlorella* species for *P. bursaria* is not based on sugar residues of their cell wall or the alkali-insoluble part of their cell wall, but rather on their ability to localize just beneath the host cell membrane soon after escaping from the host DVs.

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References

Allen AK, Neuberger A, Sharon N (1973) The purification, composition and specificity of wheat-germ agglutinin. Biochem J 131: 155–162

Bomford R (1965) Infection of alga-free *Paramecium bursaria* with strains of *Chlorella*, Scenedesmus, and a yeast. J Protozool 12: 221–224

Dryl S (1959) Antigenic transformation in *Paramecium aurelia* after homologous antiserum treatment during autogamy and conjugation. J Protozool 6 Suppl: 25

Ebisu S, Goldstein IJ (1978) *Bandeiraea simplicifolia* lectin II. In: Ginsburg V (ed) Complex carbohydrates, part C. Methods in enzymology, vol 50. Academic Press, London, pp 350–354

Goldstein IJ, Reichert CM, Misaki A (1974) Interaction of concanavalin A with model substrates. Ann N Y Acad Sci 234: 283–296

Goldstein IJ, Hammarstrom S, Sundblad G (1975) Precipitation and carbohydrate-binding specificity studies on wheat germ agglutinin. Biochim Biophys Acta 405: 53–61

Gu FK, Chen L, Ni B, Zhang X (2002) A comparative study on the electron microscopic enzymo-cytochemistry of *Paramecium bursaria* from light and dark cultures. Eur J Protistol 38: 267–278

Hiwatashi K (1968) Determination and inheritance of mating type in *Paramecium caudatum*. Genetics 58: 373–386

Hosoya H, Kimura K, Matsuda S, Kitaura M, Takahashi T, Kosaka T (1995) Symbiotic algae-free strains of the green paramecium *Paramecium bursaria* produced by herbicide paraquat. Zool Sci 12: 807–810

Jennings HS (1938) Sex reaction types and their interrelations in *Paramecium bursaria*. I and II. Clones collected from natural habitats. Proc Natl Acad Sci USA 24: 112–120

Karakashian MW (1975) Symbiosis in *Paramecium bursaria*. Symp Soc Exp Biol 29: 145–173

Karakashian SJ (1963) Growth of *Paramecium bursaria* as influenced by the presence of algal symbionts. Physiol Zool 36: 52–68

Karakashian SJ, Karakashian MW (1965) Evolution and symbiosis in the genus *Chlorella* and related algae. Evolution 19: 368–377

Karakashian SJ, Rudzinska MA (1981) Inhibition of lysosomal fusion with symbiont-containing vacuoles in *Paramecium bursaria*. Exp Cell Res 131: 387–393

Kodama Y, Fujishima M (2005) Symbiotic *Chlorella* sp. of the ciliate *Paramecium bursaria* do not prevent acidification and lysosomal fusion of the host digestive vacuoles in infection. Protoplasma 225: 191–203

Kodama Y, Nakahara M, Fujishima M (2007) Symbiotic alga *Chlorella vulgaris* of the ciliate *Paramecium bursaria* shows temporary resistance to host lysosomal enzymes during the early infection process. Protoplasma 230: 61–67

Lyer PN, Wilkinson KD, Goldstein LJ (1976) An N-acetyl-D-glycosamine binding lectin from *Bandeiraea simplicifolia* seeds. Arch Biochem Biophys 177: 330–333

Meier R, Wiessner W (1989) Infection of algae-free *Paramecium bursaria* with symbiotic *Chlorella* sp. isolated from green paramecia. II. A timed study. J Cell Sci 93: 571–579

Nishihara N, Takahashi T, Kosaka T, Hosoya H (1996) Characterization of endosymbiotic algae in *Paramecium bursaria*. Jpn J Protozool 29: 35 (in Japanese)

Ogata S, Muramatsu T, Kobata A (1975) Fractionation of glycopeptides by affinity column chromatography on concanavalin A-sepharose. J Biochem 78: 687–696

Pado R (1965) Mutual relation of protozoans and symbiotic algae in *Paramecium bursaria.* I. The influence of light on the growth of symbionts. Folia Biol 13: 173–182

Poretz RD, Goldstein IJ (1970) An examination of the topography of the saccharide binding sites of concanavalin A and of the forces involved in complexation. Biochemistry 9: 2890–2896

Reisser W (1976) Die stoffwechselphysiologischen Beziehungen zwischen *Paramecium bursaria* Ehrbg. und *Chlorella* spec. in der *Paramecium bursaria*-Symbiose. I. Der Stickstoff- und der Kohlenstoff-Stoffwechsel. Arch Microbiol 107: 357–360

Reisser W, Radunz A, Wiessner W (1982) Participation of algal surface structures in the cell recognition process during infection of aposymbiotic *Paramecium bursaria* with symbiotic chlorellae. Cytobios 33: 39–50

Ryter A, Hellio R (1980) Electron-microscope study of *Dictyostelium discoideum* plasma membrane and its modifications during and after phagocytosis. J Cell Sci 41: 75–88

Takeda H, Hirokawa T (1978) Studies on the cell wall of *Chlorella.* I. Quantitative changes in cell wall polysaccharides during the cell cycle of *Chlorella ellipsoidea*. Plant Cell Physiol 19: 591–598

Takeda H, Sekiguchi T, Nunokawa S, Usuki I (1998) Species-specificity of *Chlorella* for establishment of symbiotic association with *Paramecium bursaria*. Does infectivity depend upon sugar components of the cell wall? Eur J Protistol 34: 133–137

Weis DS (1969) Regulation of host and symbiont population size in *Paramecium bursaria*. Experientia 15: 664–666

Weis DS (1980) Hypothesis: Free maltose and algal cell surface sugars are signals in the infection of *Paramecium bursaria* by algae. In: Schwemmler W, Schenk EA (eds) Endosymbiosis and cell biology, vol I. Walter de Gruyter, Berlin, pp 105–112

Weis DS, Ayala A (1979) Effect of exposure period and algal concentration on the frequency of infection of aposymbiotic ciliates by symbiotic algae from *Paramecium bursaria*. J Protozool 26: 245–248

Wichterman (1948) The biological effects of X-rays on mating types and conjugation of *Paramecium bursaria*. Biol Bull 93: 201

Yamamoto K, Tsuji T, Matsumoto I, Osawa T (1981) Structural requirements for the binding of oligosaccharides and glycopeptides to immobilized wheat germ agglutinin. Biochemistry 29: 5894–5899