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| Author(s) | Higuchi, Rina / Song, Chihong / Hoshina, Ryo / Suzaki, Toshinobu |
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Endosymbiosis-related changes in ultrastructure and chemical composition of *Chlorella variabilis* (Archaeplastida, Chlorophyta) cell wall in *Paramecium bursaria* (Ciliophora, Oligohymenophorea)

Rina Higuchi\(^a\), Chihong Song\(^a,b\), Ryo Hoshina\(^c\), Toshinobu Suzaki\(^a,\ast\)

\(^a\)Department of Biology, Graduate School of Science, Kobe University, Nada-ku, Kobe 657-8501, Japan
\(^b\)National Institute for Physiological Sciences, 5-1 Higashiyama, Myodaiji, Okazaki, Aichi, 444-8787, Japan
\(^c\)Nagahama Institute of Bioscience and Technology, Nagahama, Japan

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Abstract

*Chlorella variabilis*, a symbiotic alga, is usually present in the cytoplasm of *Paramecium bursaria*, although it can be cultured in host-free conditions. Morphological and chemical properties of its cell wall were compared between its free-living and symbiotic states. Transmission electron microscopy (quick-freezing and freeze-substitution methods) revealed that the cell wall thickness of symbiotic *C. variabilis* was reduced to about half that of the free-living one. Chemical properties of the cell wall were examined by treatment with three fluorescent reagents (calcofluor white M2R, FITC-WGA, and FITC-LFA) having specific binding affinities to different polysaccharides. When the algae were re-introduced into *Paramecium* host cells, calcofluor fluorescence intensity reduced by about 50%. Calcofluor stains β-D-glucopyranose polysaccharides such as cellulose, N-acetylglucosamine, sialic acid, and glycosaminoglycans. Because treatment with cellulase showed no effect on calcofluor fluorescence intensity, we considered that cellulose is not majorly responsible for the stainability of calcofluor. Staining intensities of FITC-WGA and FITC-LFA were similar in the free-living and symbiotic conditions, suggesting that N-acetylglucosamine and sialic acid are not responsible for the reduction in the stainability of calcofluor associated with intracellular symbiosis. The amount of glycosaminoglycans on the cell wall may decrease in *C. variabilis* present in the cytoplasm of *P. bursaria*.

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Keywords: Cell wall; *Chlorella variabilis*; Glycosaminoglycans; *Paramecium bursaria*; Symbiosis

Introduction

Hundreds of *Chlorella* cells may be present in the cytoplasm of a single *Paramecium bursaria*. The host P. bursaria supplies nitrogen sources (Albers and Wiessner 1985) and CO\(_2\) (Reisser 1980) to *C. variabilis*, and while living inside the host cells, *C. variabilis* seems to be able to avoid infection by *Chlorella* virus (Kawakami and Kawakami 1978). The symbiont, on the other hand, supplies maltose, a photosynthetic product, to the host (Zieseniz et al. 1981). In addition, the host *P. bursaria* shows an enhanced tolerance to high temperature and UV due to the presence of the symbiotic *Chlorella* (Iwatsuki et al. 1998; Summerer et al. 2009). Thus, *P. bursaria* and *C. variabilis* are considered to be in a mutually beneficial symbiotic relationship. However, this symbiotic relationship is not an obligatory one; the symbiont can be artificially removed from the host (Reisser 1976), and...
aposymbiotic white paramecia can grow in the same manner as ordinary green ones. Isolated *C. variabilis* can be cultured alone, and when white *P. bursaria* and isolated *C. variabilis* are mixed together, *C. variabilis* is ingested by white *P. bursaria*, and the symbiotic relationship resumes within a few days (Ziesenisz et al. 1981). From these observations, it has been considered that *P. bursaria* is in the very early stage of secondary symbiosis and is suitable as a model system to elucidate the mechanism of intracellular symbiosis. Despite various studies to date, however, the molecular basis involved in establishing and maintaining intracellular symbiosis in *P. bursaria* remains unclear. Many of the previous studies have focused on the fine structure and gene expression of host cells (Kodama et al. 2014; Song et al. 2017), but there have been only few approaches focusing on the symbiont. The only report related to this showed that a component extracted from *P. bursaria* cells enhanced *Chlorella*’s carbon fixation ability and activated photosynthesis (Kamako and Imamura 2006). Working from this perspective, this research aimed to analyze changes in the structure and composition of the cell wall of *C. variabilis* accompanying intracellular symbiosis in *P. bursaria*.

**Material and Methods**

**Ciliate and algal cultures**

*P. bursaria* was initially collected from a pond in Kobe, Japan, and was cloned before use in this study. Aposymbiotic (white) *P. bursaria* was produced by mixing the cloned green *P. bursaria* with 0.1% (w/v) paraquat (methyl viologen: Sigma, M-2254) according to the method described by Tanaka et al. (2002). Both *P. bursaria* and aposymbiotic *P. bursaria* were cultured monoxenically with *Chlorogonium capitatum* (NIES-3374) as the sole food source (Omura et al. 2004). Symbiotic *Chlorella* cells were isolated by gently rupturing the cell membrane of *P. bursaria* with an ultrasonic disruptor (TOMY, UR-20P) for 1 min at room temperature. The *Chlorella* cells obtained were cultured on an agar plate containing medium supplemented with 0.03% (w/v) L-serine (Kato and Imamura 2008) and were cloned using a single colony. The resulting strain (named Kb1, Fig. 1B) was cultured for more than 1 month in liquid C medium with L-serine and re-introduced into an aposymbiotic *P. bursaria* to yield a Kb1-bearing green *P. bursaria*, which was named as the Pb-Kb1 strain (Fig. 1A). The re-constructed Pb-Kb1 strain was cultured for more than one year before being used for experiments.

**Transmission electron microscopy**

Quick-freezing and freeze-substitution methods were employed for transmission electron microscopy because these methods proved to be superior to conventional chemical fixation for the preservation of the fine structures of the *Chlorella* cell wall (Song and Suzuki 2013; Song et al. 2017). *Chlorella* cells were harvested by low-speed centrifugation and re-suspended at a high cell density before cryofixation by impact-freezing onto a liquid nitrogen-cooled copper block using a freezing device (VFZ-1, Japan Vacuum Device, Ltd., Japan). The specimens were freeze-substituted in acetone containing 1% OsO₄ for 3 days. They were then brought back to room temperature and embedded in Spurr’s resin (Polysciences, Inc., Warrington, PA). Ultrathin sections (100 nm) were cut with a diamond knife, stained with EM Stainer (Nissin EM, Ltd., Tokyo, Japan) and Reynolds’ lead citrate stain (Reynolds 1963), and observed using a transmission electron microscope (Hitachi H-7100, Hitachi Ltd., Tokyo, Japan).

**Measurement of fluorescence intensity by confocal laser scanning microscopy**

To investigate whether the chemical composition of *Chlorella* cell walls changes due to intracellular symbiosis to *Paramecium*, free and symbiotic *Chlorella* cells were stained with sugar-binding fluorescent reagents, and their staining intensities were compared.

Three sugar-binding reagents were used for fluorescent staining of the cell surface of *Chlorella* cells: calcofluor White M2R (Fluorescent Brightener, Sigma, St Louis, MO), FITC-labeled WGA (EY Laboratories, San Mateo, CA, USA), and FITC-labeled LFA (EY Laboratories). The sugar compositions recognized by these reagents are different and are summarized in Table 1. First, *Chlorella* cells were centrifuged at 5000 rpm for 5 min at 20°C, and the supernatant was discarded. Then, for calcofluor staining, an aqueous solution of the reagent (0.05%, w/v) was added to the specimen and stained for 5 min at 20°C. For staining with WGA and LFA, *Chlorella* cells were mixed with 0.01% (w/v) of the reagents for 10 min at 20°C. Thereafter, the samples were washed three times with distilled water, mixed with the same amount of anti-fading agent Aqua-Poly/Mount (Polysciences), and then placed on a glass slide and observed using a confocal laser scanning microscope (CLSM) (Olympus, FV-1000, Tokyo, Japan).

To examine whether cellulose is involved in calcofluor staining, an experiment was conducted using cellulase (cat. no. 07550-74, Nacalai Tesque Inc., Kyoto, Japan). Free-living and symbiotic *Chlorella* cells were treated with cellulase at 1%, 2%, or 5% (w/v) for 24 h at 20°C. The specimens were then washed three times with distilled water and stained with calcofluor as described above. The excitation/emission wavelength combinations of the reagents used in CLSM are also listed in Table 1. The autofluorescence (ex: 405 nm, em: 630–730 nm) of *Chlorella* was used as a criterion for judging that the cells were alive and healthy.

Quantitative analysis of fluorescence intensity was performed using the image analysis software Image J (NIH) for
Fig. 1. Light micrographs of Paramecium bursaria strain Pb-Kb1 (A) and isolated Chlorella variabilis strain Kb1(B). Scale bars are 10 μm in A and 2 μm in B.

Table 1. Fluorescent reagents used in this study.

| Possible target molecules in Chlorella cell walls | Excitation/emission wavelength (nm) |
|-----------------------------------------------|-----------------------------------|
| Sialic acid                                   | 473/485–585                       |
| Chitin                                        |                                    |
| Cellulose                                     |                                    |
| Glycosaminoglycans                            |                                    |
| 405/525–545                                   |                                    |
| 473/485–585                                   |                                    |
| 473/485–585                                   |                                    |

Calcofluor is a fluorescent dye that reacts with β-n-glucopyranose polysaccharide (Chen et al. 2007), including cellulose, chitin, sialic acid, and glycosaminoglycans (Bezares et al. 2008; Albani et al. 2000; Kapaun and Reisser 1995). WGA is a lectin that binds to N-acetylgalactosamine, chitin, and sialic acid (Rodrigues et al. 2008). LFA is also a lectin that specifically reacts with sialic acid (Fischer and Brossmer 1995).

fluorescence images taken by CLSM. First, the viability of Chlorella cells was evaluated based on the autofluorescence of chlorophyll. Living cells were selected, and fluorescence images of calcofluor-, WGA-, or LFA-stained cells were obtained. An area that covered a stained cell was designated, and the average brightness of the area was determined using Image J.

PCR and sequencing of Kb1

DNA extractions using the NucleoSpin Plant II Kit (Macherey-Nagel, Düren, Germany) was performed. Polymerase chain reaction (PCR) was carried out to amplify ITS (Internal Transcribed Spacer) rDNA by using the primer pairs INT-4F (Hoshina et al. 2004)/HLR-3R (Hoshina et al. 2005). PCR product was purified with NucleoSpin Gel and PCR Clean-up (Macherey-Nagel, Düren, Germany), and was directly sequenced on an ABI PRISM 3100 Genetic Analyzer (Applied Biosystems, Foster City, CA) using BigDye Terminator chemistry (Applied Biosystems).

Results

Identification of Kb1

The sequence of Kb1 (1263 bp) was obtained (GenBank Accession No., LC420163), which includes ITS1–5.8S–ITS2 rDNA and a group I intron at position L200. The ITS2 sequence of Kb1 was identical to several sequences of Chlorella variabilis including the authentic strain SAG 211-6 and the most famous strain ATCC 50258/CCAP 211/84 (NC64A). The sequence was separated from the other species (Fig. 2), therefore, Kb1 can be identified as C. variabilis. As a side note, the L200 introns have been found only from C. variabilis (Hoshina and Imamura 2009).

Thickness and fine structure of the cell wall of free-living and symbiotic C. variabilis

Free-living (cultured alone for >1 month) and symbiotic Chlorella living in P. bursaria were observed using a transmission electron microscope. Twenty-one individuals of free-living and symbiotic C. variabilis were randomly selected, and the thickness of the cell wall was measured at an arbitrary position of each cell. Because the outer surface of the cell wall showed a fluffy appearance (Fig. 3B and C), the thickness of the cell wall was measured as the distance from the base of the fluffy structure to the innermost position of the cell wall. The thickness of the cell wall of the free-living C. variabilis was 11–17 nm and that of the symbiotic C. variabilis was 6–10 nm (Fig. 3A).

The fluffy structure was observed on the surface of cell walls in both free-living and symbiotic C. variabilis cells. The fluffy filaments have a uniform length of about 15 nm.
Changes in carbohydrate composition in the cell wall

Fluorescence intensity of calcofluor-stained cells was measured on free-living and symbiotic C. variabilis cells. The cell wall was stained with calcofluor, which is known to react with sialic acid, chitin, cellulose, and glycosaminoglycans (Table 1). The cell wall in the endosymbiotic state showed weaker fluorescence as compared with that in the free-living state (Fig. 4), apparently indicating a decrease in a calcofluor-reacting carbohydrate as a result of symbiosis. Staining intensities of two types of lectins (LFA and WGA) were also examined to further characterize the molecular composition in the cell wall. These lectins also stained the cell wall well, but unlike calcofluor, no significant changes were detected between free-living and symbiotic cells (Fig. 5), indicating that chitin and sialic acid may not account for the reduction of calcofluor staining that accompanies endosymbiosis.

To examine the possible involvement of cellulose in calcofluor staining, free-living C. variabilis was treated with different concentrations of cellulase for 24 h. The cellulase-treated cells were then subjected to calcofluor staining and examined by CLSM. As shown in Fig. 5C, the fluorescence
Bars show the composition of calcofluor stained in Fig. 3. Comparison of cell wall thickness and ultrastructure of *C. variabilis* in free-living and symbiotic states. The cell wall of *C. variabilis* in the free-living state was about 2 times thicker than that in the symbiotic state (A). Error bars in A represent standard deviations. Welch’s t test was used for statistical analysis (n = 21), which showed a significant difference (p < 0.01). Transmission electron micrographs in B and C show cross sections of the cell wall of free-living and symbiotic *C. variabilis* cells, respectively. w: cell wall, m: *Chlorella* cell membrane. The surface of cell walls in both free-living *C. variabilis* (B) and symbiotic *C. variabilis* (C) appears to be covered with a fine fibrous layer. Bars in B and C represent 100 nm.

The fluorescence intensity was not influenced by cellulase treatment, even at a very high concentration (5%, at which complete degradation of plant cell walls usually occurs). These results suggested that cellulose is not a major sugar component stained by calcofluor and that it may be glycosaminoglycans that are responsible for the reduction of calcofluor staining in the endosymbiotic *C. variabilis* cells.

**Discussion**

In this study, we compared the ultrastructure and chemical composition of the cell wall of *C. variabilis* in free-living and symbiotic states. To establish a stable coexistence within the cytoplasm of *P. bursaria, Chlorella* cells need to avoid lysosomal attacks by host cells (Dolan 1992). The symbiotic *Chlorella* within the host cell is enclosed by a membrane called the peri-algal vacuole (PV) membrane (Karakashian et al. 1968; Meier et al. 1984). Using a novel cryofixation technique, we have recently found that the PV membrane is closely attached to the tips of filamentous appendages on the outer surface of the cell wall of symbiotic *C. variabilis* strain Kb1 cells (Song and Suzaki 2013). We also reported that mitochondria of host cells frequently form intimate connections with the symbiont cell wall via the PV membrane (Song et al. 2017). These observations suggest the importance of the interaction between the *Chlorella* cell wall and the PV membrane in the formation and maintenance of the symbiotic relationship.

Observation by electron microscopy revealed that the cell wall of *Chlorella* became thinner in the symbiotic condition. In *C. vulgaris*, it has been reported that the cell wall thickness remains constant even when culture conditions are changed (Martínez et al. 1991). To the authors’ knowledge, there are no reports stating that the thickness of the cell wall changes depending on environmental conditions. The decrease in the cell wall associated with symbiosis found in this study may be attributed to the result of the symbiotic interaction between the host and symbiont. Because the main function of the algal cell wall is to provide structural support and protection against mechanical stress, it is not necessary for endosymbiotic algal cells to retain their rigid cell walls in the endosymbiotic condition, and reduction of cell wall thickness in endosymbiosis may be a reasonable consequence.

In the present study, we showed that the staining intensity of calcofluor in the cell wall of *C. variabilis* changes owing to symbiosis. Because calcofluor is a fluorescent dye that reacts with β-D-glucopyranose polysaccharide (Chen et al. 2007), our results indicate that the amount of β-D-glucopyranose polysaccharide on *Chlorella* cell wall might have reduced in the symbiotic state. Therefore, we examined the amount...
of chitin, sialic acid, and glycosaminoglycans, which can be stained with calcofluor (Bezares et al. 2008; Albani et al. 2000; Kapaun and Reisser 1995) and which are known to be present in the Chlorella cell wall (Kapaun and Reisser 1995; Correll 1964). Because calcofluor stains cellulose well, we also assessed the possibility of the presence of cellulose in the Chlorella cell wall. As shown in Fig. 5, the experiment involving cellulase digestion demonstrated that calcofluor staining might not be attributed to the presence of cellu-
lose. Although it is a conjecture based on the elimination method, our experiments demonstrated that glycosaminoglycans remained as the only possible substances considered to be the cause of reduction in calcofluor stainability among substances that can be stained by calcofluor. Glycosaminoglycans are polysaccharides composed of sugars to which sulfate groups are added, including chondroitin sulfate and hyaluronic acid. Karakashian and Rudzinska (1981) sug-
gested that some polyanionic component present in the cell wall of Chlorella aids in preventing lysosomal attack to the PV membrane. Glycosaminoglycans are negatively-charged polyanionic polysaccharide chains, and it is therefore plausible that glycosaminoglycans in the Chlorella cell wall may pass through the PV membrane, thereby acting to avoid lysosomes from the PV membrane. To fully understand such an avoidance mechanism, molecular characterization of the PV membrane would be necessary. Takeda et al. (1998) reported that the sugar composition of the cell walls differs between symbiotic and free-living species of Chlorella: symbiotic species are rich in glucosamine, one of the component sug-
ars of glycosaminoglycans. A glycoprotein on the cell wall of a symbiotic alga has been reported to be important for establishing symbiosis with sea anemone (Lin et al. 2000). When Legionella bacteria infects animal cells, it releases vesicles made of lipopolysaccharide inside the phagosome and inhibits fusion by lysosomes (Fernandez-Moreira et al. 2006). Based on these similarities and our results, cell wall polysaccharides are suggested to be important in facilitating intracellular symbiosis of C. variabilis in the cytoplasm of P. bursaria.

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References

Albani, J.R., Sillen, A., Plancke, Y.D., Coddeville, B., Engelborghs, Y., 2000. Interaction between carbohydrate residues of α1-acid glycoprotein (orosomucoid) and saturating concentrations of Calcofluor White. A fluorescence study. Carbohydr. Res. 327, 333–340.

Albers, D., Wiessner, W., 1985. Nitrogen nutrition of endosymbiotic Chlorella spec. Endocytobiol. Cell Res. 2, 55–64.

Bezares, J., Asaro, R.J., Hawley, M., 2008. Macromolecular structure of the organic framework of nacre in Haliotis rufescens: implications for growth and mechanical behavior. J. Struct. Biol. 163, 61–75.

Chen, M.Y., Lee, D.J., Tay, J.H., 2007. Distribution of extracellular polymeric substances in aerobic granules. Appl. Microbiol. Biotechnol. 73, 1463–1469.

Correll, D.L., 1964. Sialic acid-containing glycopeptide from Chlorella. Science 145, 588–589.

Dolan, J., 1992. Mixotrophy in ciliates: a review of Chlorella sym-
biosis and chloroplast retention. Mar. Microb. Food Webs 6, 115–132.

Fernandez-Moreira, E., Helbig, J.H., Swanson, M.S., 2006. Membrane vesicles shed by Legionella pneumophila inhibit fusion of phagosomes with lysosomes. Infect. Immun. 74, 3285–3295.

Fischer, E., Brossmer, R., 1995. Sialic acid-binding lectins: sub-
molecular specificity and interaction with sialoglycoproteins and tumour cells. Glycoconjug. J. 12, 707–713.

Heeg, J.S., Wolf, M., 2015. ITS2 and 18S rDNA sequence-structure phylogeny of Chlorella and allies (Chlorophyta, Trebouxiophyceae, Chlorellaceae). Plant Gene 4, 20–28.
Hoshina, R., Imamura, N., 2009. Phylogenetically close group I introns with different positions among *Paramecium bursaria* photobionts imply a primitive stage of intron diversification. Mol. Biol. Evol. 26, 1309–1319.

Hoshina, R., Kamako, S., Imamura, N., 2004. Phylogenetic position of endosymbiotic green algae in *Paramecium bursaria* Ehrenberg from Japan. Plant Biol. 6, 447–453.

Hoshina, R., Kato, Y., Kamako, S., Imamura, N., 2005. Genetic evidence of “American” and “European” type symbiotic algae of *Paramecium bursaria* Ehrenberg. Plant Biol. 7, 526–532.

Iwatsuki, K., Nishidou, M., Suehiro, K., 1998. Symbiotic *Chlorella* enhances the thermal tolerance in *Paramecium bursaria*. Comp. Biochem. Phys. A 121, 405–409.

Kamako, S., Imamura, N., 2006. Effect of Japanese *Paramecium bursaria* extract on photosynthetic carbon fixation of symbiotic algae. J. Eukaryot. Microbiol. 53, 136–141.

Kapaun, E., Reisser, W., 1995. A chitin-like glycan in the cell wall of a *Chlorella* sp. (Chlorococcales, Chlorophyceae). Planta 197, 577–582.

Karakashian, S.J., Rudzinska, M.A., 1981. Inhibition of lysosomal fusion with symbiotic-containing vacuoles in *Paramecium bursaria*. Exp. Cell Res. 131, 387–393.

Karakashian, S.J., Karakashian, M.W., Rudzinska, M.A., 1968. Electron microscopic observations on the symbiosis of *Paramecium bursaria* and its intracellular algae. J. Protozool. 15, 113–128.

Kato, Y., Imamura, N., 2008. Effect of calcium ion on uptake of amino acids by symbiotic *Chlorella* F36-ZK isolated from Japanese *Paramecium*. Plant Sci. 174, 88–96.

Kawakami, H., Kawakami, N., 1978. Behavior of a virus in a symbiotic system, *Paramecium bursaria* – zoochlorella. J. Protozool. 25, 217–225.

Kodama, Y., Suzuki, H., Dohra, H., Sugii, M., Kitazume, T., Yamaguchi, K., Shigenobu, S., Fujishima, M., 2014. Comparison of gene expression of *Paramecium bursaria* with and without *Chlorella variabilis* symbionts. BMC Genomics 15, 183.

Larkin, M.A., Blackshields, G., Brown, N.P., Chenna, R., McGettigan, P.A., McWilliam, H., Valentin, F., Wallace, I.M., Wilm, A., Lopez, R., Thompson, J.D., Gibson, T.J., Higgins, D.G., 2007. Clustal W and Clustal X version 2.0. Bioinformatics 23, 2947–2948.

Lin, K.L., Wang, J.T., Fang, L.S., 2000. Participation of glycoproteins on zooxanthellae cell walls in the establishment of a symbiotic relationship with the sea anemone, *Aiptasia pulchella*. Zool. Stud. 39, 172–178.

Martínez, F., Ascaso, C., Orús, M.I., 1991. Morphometric and stereological analysis of *Chlorella vulgaris* under heterotrophic growth conditions. Ann. Bot-London 67, 239–245.

Meier, R., Lefort-Tran, M., Poupilhe, M., Reisser, W., Wiessner, W., 1984. Comparative freeze-fracture study of perialgal and digestive vacuoles in *Paramecium bursaria*. J. Cell Sci. 71, 121–140.

Omur, G., Ishida, M., Arikawa, M., Khan, S.M.M.K., Suehiro, Y., Kakuta, S., Yoshimura, C., Suzaki, T., 2004. A bacteria-free monoxenic culture of *Paramecium bursaria*: its growth characteristics and the re-establishment of symbiosis with *Chlorella* in bacteria-free conditions. Jpn. J. Protozool. 37, 139–150.

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

Reisser, W., 1980. The metabolic interactions between *Paramecium bursaria* Ehrenbg. and *Chlorella* spec. in the *Paramecium bursaria*-symbiosis. III. The influence of different CO2-concentrations and of glucose on the photosynthetic and respiratory capacity of the symbiotic unit. Arch. Microbiol. 125, 291–293.

Reynolds, E.S., 1963. The use of lead citrate at high pH as an electron-opaque stain in electron microscopy. J. Cell Biol. 17, 208–212.

Rogrigues, M.L., Alvarenga, M., Fonseca, F.L., Casadevall, A., 2008. Binding of the Wheat Germ Lectin to *Cryptococcus neoformans* suggests an association of chitinlike structures with yeast budding and capsular glucuronoxymannan. Eukaryot. Cell 7, 602–609.

Song, C., Suzaki, T., 2013. Improved preservation of organelles in *Paramecium bursaria* by freeze-substitution with glutaraldehyde and osmium tetroxide. J. Electr. Microsc. Technol. Med. Biol. 27, 1–8.

Song, C., Murata, K., Suzaki, T., 2017. Intracellular symbiosis of algae with possible involvement of mitochondrial dynamics. Sci. Rep. 7, 1221.

Summerer, M., Sonntag, B., Hörtnagl, P., Sommaruga, R., 2009. Symbiotic ciliates receive protection against UV damage from their algae: a test with *Paramecium bursaria* and *Chlorella*. Protist 160, 233–243.

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.

Tanaka, M., Murata, H.M., Kadono, T., Yamada, T., Kawano, T., Kosaka, T., Hosoya, H., 2002. Complete elimination of endosymbiotic algae from *Paramecium bursaria* and its confirmation by diagnostic PCR. Acta Protozool. 41, 255–261.

Ziesenisz, E., Reisser, W., Wiessner, W., 1981. Evidence of de novo synthesis of maltose excreted by the endosymbiotic *Chlorella* from *Paramecium bursaria*. Planta 153, 481–485.