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(Citation)
European Journal of Protistology,66:149-155

(Issue Date)
2018-10

(Resource Type)
journal article

(Version)
Version of Record

(URL)
https://hdl.handle.net/20.500.14094/90005469

Available online at www.sciencedirect.com

ScienceDirect

European Journal of Protistology 66 (2018) 149–155

European Journal of PROTISTOLOGY

www.elsevier.com/locate/ejop

Endosymbiosis-related changes in ultrastructure and chemical composition of *Chlorella variabilis* (Archaeplastida, Chlorophyta) cell wall in *Paramecium bursaria* (Ciliophora, Oligohymenophorea)

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Received 18 July 2018; received in revised form 8 September 2018; accepted 13 September 2018
Available online 19 September 2018

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 consider 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 also 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*.

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 (Ziesenisz 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

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https://doi.org/10.1016/j.ejop.2018.09.003

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 aspect 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 capillatum* (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 C 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 Suzaki 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% OsO_4 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 (Nisshin 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

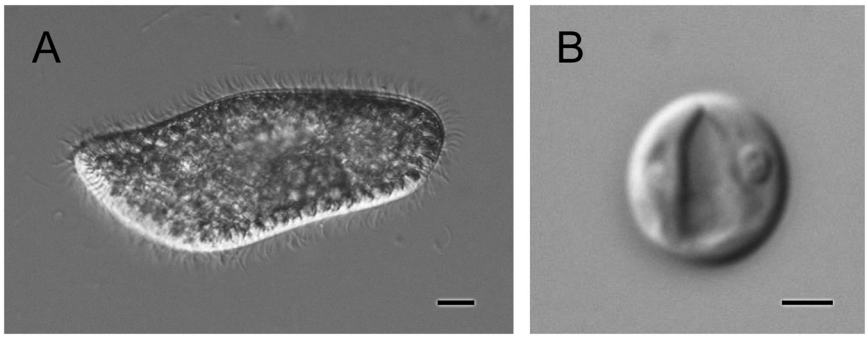

Fig. 1. Light micrographs of *Paramecium bursaria* strain Pb-Kb1 (A) and isolated *Chlorella variabilis* strain Kb1(B). Scale bars are 10 μm in A and 2 μm in B.

Table 1. Fluorescent reagents used in this study.

	Calcofluor white	FITC-WGA	FITC-LFA
Possible target molecules in *Chlorella* cell walls	Sialic acid Chitin Cellulose Glycosaminoglycans	Sialic acid Chitin	Sialic acid
Excitation/emission wavelength (nm)	405/525–545	473/485–585	473/485–585

Calcofluor is a fluorescent dye that reacts with β-D-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-acetylglucosamine, 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.

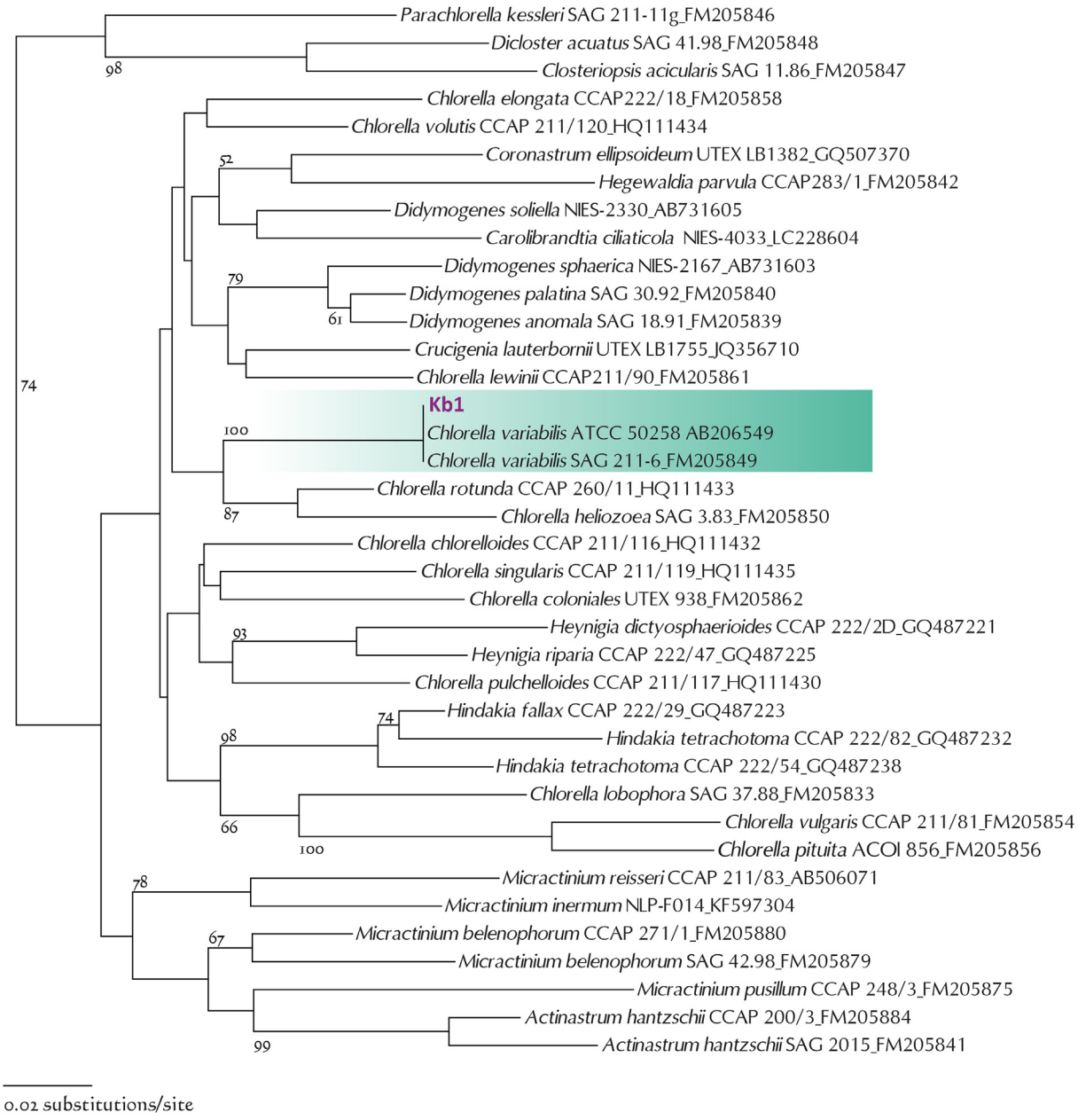

Fig. 2. Neighbor-joining tree based on ITS2 sequences. The tree was constructed via ClustalX2 (Larkin et al. 2007) program with the option of Exclude positions with gaps. The alignment was based on that of Heeg and Wolf (2015). Numbers at each node represent bootstrap probabilities of the NJ analysis; only values above 50% are shown.

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

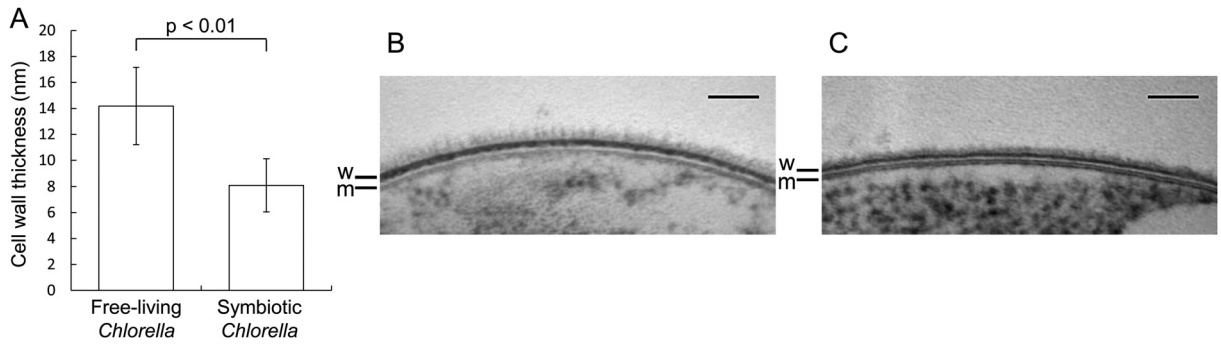

Fig. 3. Comparison of cell wall thickness and ultrastructure of *C. variabilis* in free-living and symbiotic states. The cell wall of *C. variabilis* in the free-living state was about 2 times thicker than that in the symbiotic state (A). Error bars in A represent standard deviations. Welch'st test was 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.

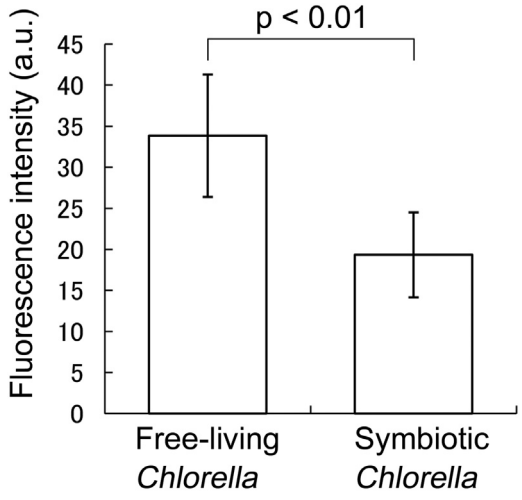

Fig. 4. Comparison of fluorescence intensity of cell walls after calcofluor staining. Free-living and symbiotic *C. variabilis* cells were stained with calcofluor, and the fluorescence intensity, represented in arbitrary units (a. u.), was compared. The fluorescence intensity of symbiotic cells (n = 27) was significantly decreased as compared with that of free-living cells (n = 47). Error bars represent standard deviations. Welch'st test was used for statistical analysis.

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

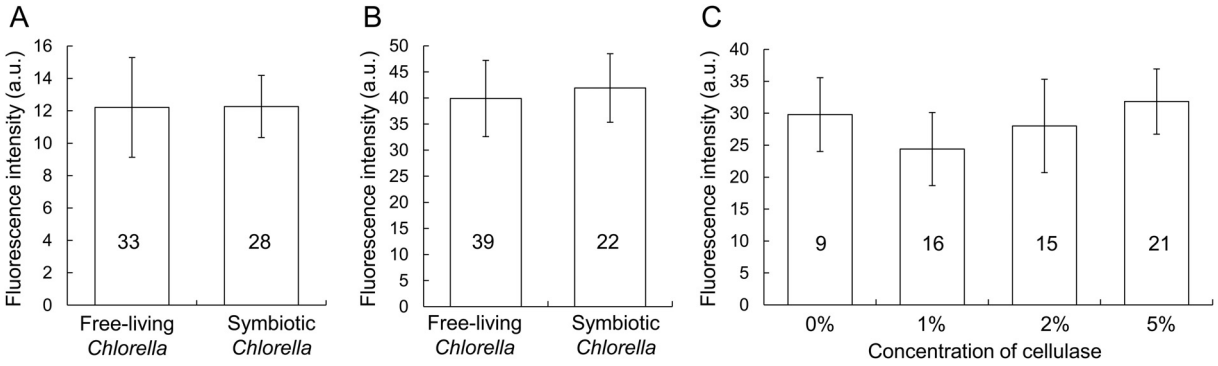

Fig. 5. Comparison of fluorescence intensities of free-living and symbiotic *Chlorella variabilis* cells measured after staining with FITC-LFA (A) or FITC-WGA (B), and the fluorescence intensity of calcofluor of *Chlorella* pretreated with different concentrations of cellulase (C). In both LFA- and WGA-stained *Chlorella* cells, fluorescence intensities were not significantly different between free-living and symbiotic conditions. Cellulase treatment did not result in changes in calcofluor staining (C). Error bars show standard deviations, and Welch'st test was used for statistical analysis. The numbers in the bars represent sample numbers.

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 cellulose. 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) suggested 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 sugars 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.*

Acknowledgements

Ryo Hoshina is financially aided by Japan Society for the Promotion of Science KAKENHI Grant Number 16K07491.

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