**Colony sheath formation is accompanied by shell formation and release in the
green alga *Botryococcus braunii* (Race B)**

Yuki Uno^a, Ichiro Nishii^{a,b,c}, Satoshi Kagiwada^{a,b,c}, Tetsuko Noguchi^{a,b,c,#}

^aGraduate School of Humanities and Sciences, Nara Women's University, Nara
630-8506, Japan

^bCourse of Biological Sciences, Faculty of Science, Nara Women's University, Nara
630-8506, Japan

^cJapan Science and Technology Agency, CREST, 5 Sanbancho, Chiyoda, Tokyo
102-0075, Japan

(Running title: Colony sheath formation in *Botryococcus*)

[#]Corresponding author's e-mail: noguchi@cc.nara-wu.ac.jp

Course of Biological Sciences, Faculty of Science, Nara Women's University, Nara
630-8506, Japan.

TEL & FAX; 81-742-20-3416

27 Abstract

28 *Botryococcus braunii* race B is a promising candidate for renewable biofuel
29 production. Its colony organization is unique. Cells are connected by extracellular
30 biopolymers containing hydrocarbons at their basolateral regions, enclosed by a
31 retaining wall, and form a sphere inside a colony sheath composed of polysaccharide
32 fibrils. This unique colony structure is a key for the hydrocarbon production and
33 storage, but how the colony forms remains unknown. We studied ultrastructure of
34 colony sheath and its formation process by rapid freezing and freeze-substitution
35 electron microscopy. Fibrils of colony sheath extend from the retaining wall and are
36 branched, and repel India ink. The sheath formation starts in mitosis and completes
37 during the following daughter cell maturation. In this process, the older retaining wall
38 and thin layers at the cell apex are released as shells and new fibrils formed on the
39 new retaining wall extend into and merge with the pre-existing colony sheath. Its
40 formation also accompanied by the hydrocarbon secretion and accumulation at the
41 basolateral region. The fibrils of colony sheath that extend from a cell wall are
42 replaced by new fibrils after each cell division, while fibrils in the extracellular
43 biopolymers accumulate to form the remaining part of sheath, without replacement.

44

45

46 Key words; colony sheath, electron microscopy, green alga, hydrocarbon

1. Introduction

Oil production by photosynthetic microalgae has attracted attention because of the possibility of producing renewable biofuels [1,2,3]. The potential productivity of oleaginous microalgae per unit area can be 8 to 24 times that of the best oil-producing land plants [4]. Among the oleaginous microalgae, *Botryococcus braunii* (Chlorophyta, Trebouxiophyceae) is extraordinary for two reasons: First, it synthesizes large amounts of hydrocarbons in addition to a few triacylglycerols that other algae, such as *Chlamydomonas reinhardtii* [5], *Chlorella sorokiniana* [6], and *Dunaliella salina* [7], normally produce. Second, it accumulates the hydrocarbons in extracellular spaces [8-12], whereas other microalgae that have been studied, including oleaginous species in which active oil production is induced by environmental stress, store oils in their cytoplasm [5-7]. The extracellular biopolymers containing hydrocarbons are essential for producing the unique colony form of *B. braunii* [12]. *Botryococcus braunii* is classified into three principal races (A, B, and L) based on the types of hydrocarbons it synthesizes [13,14]: race A produces alkadienes and alkatrienes (from C₂₃ to C₃₃) [15]; race B produces triterpenes, but mainly botryococcenes (from C₃₀ to C₃₇) and methylated squalenes (from C₃₁ to C₃₄) [16,17]; and race L produces tetraterpene, lycopadiene [18].

Among the microalgae that have been screened as candidates for renewable biofuel production, *B. braunii* is one of the most promising candidates. First, it produces especially large quantities of hydrocarbons [2], reaching up to 86% of its cell dry weight [19]. Second, it accumulates these compounds mainly in extracellular spaces, which facilitates lipid extraction from the algal biomass. Third, its hydrocarbons can be readily converted into biofuels, especially the triterpenoids (botryococcenes and methylated squalenes) produced by race B [2]. In addition to the third reason, race B

generally has a higher overall content of hydrocarbons than the other races [14], and thus it has attracted the most attention as a candidate that produces alternatives to petroleum.

Because of this high potential, the biosynthetic pathways that produce botryococenes and methylated squalenes have been actively studied. Race B uses the methylerythritol phosphate (MEP) pathway to produce universal precursors for isoprenoids, and three genes that encode enzymes involved in this pathway have been cloned from race B [20]. In addition, the genes that encode key enzymes (squalene synthase like enzymes; SSL-1, 2, and 3) that catalyze the final steps of the transformation from farnesyl diphosphate to C₃₀ botryococcene (SSL-1 and 3) and squalene (SSL-1 and 2) have been identified [21].

To achieve effective biofuel production using *B. braunii* race B, genetic engineering should be attempted to improve the utility of this race. As a result, genetic transformation has been attempted in many laboratories. However, no successful procedures have been reported, despite successful transformation of other microalgae [22] including green algae [23,24], red algae [25], diatoms [26], euglenoids [27] and dinoflagellates [28]. Unfortunately, the difficulty of transformation is thought to be caused by the characteristics that make *B. braunii* a promising candidate for renewable biofuel production: the large amounts of hydrocarbons deposited in the extracellular matrix might interfere with effective transfer of nucleic acids into the cell by several transformation methods.

Understanding the colony structure might provide insights into how to solve this problem. Unfortunately, ultrastructural analysis using electron microscopy had been complicated by the large amount of extracellular hydrocarbons. We have overcome this problem by developing a method of quick-freeze fixation to replace chemical

fixation for electron microscopy in race A [29-32], and have recently applied the same method to race B [12]. In race B, we discovered that each cell was covered by three to six thin layers containing hydrocarbons at the basolateral region that covered 70 to 90% of the cell surface, and that these layers held together the neighboring cells to form a colony. In addition, these cells were entirely enclosed by a fibrillar colony sheath [12].

Part of the colony sheath was first observed using thin-section electron microscopy by Largeau et al. in 1980 [8]; they called this structure the “polysaccharide fibrils”. An image of the entire colony sheath (fibrillar sheath) that encloses the colony was obtained by Weiss et al. in 2012 using quick-freeze, deep-etch electron microscopy [33]. However, thin-section electron microscopy has generally failed to clearly show the fibrils of the *B. braunii* colony sheath when samples were prepared by means of chemical fixation or even quick-freeze fixation in some cases. This problem occurred in *B. braunii* race B frozen under high pressure (210 MPa) before electron microscopy, despite the ability of this technique to produce excellent images of the cell’s organelles [12], and in race A frozen with liquid propane and treated with 2% osmium tetroxide, with which we captured clear images of the colony sheath only in a 500-nm thick section using ultra-high-voltage (2000-kV) electron microscopy, but not by using the usual 70- to 100-nm sections under an 80-kV electron microscope [32].

In this study, we improved our fixation method to reveal the fine fibrillar structure of the colony sheath and clarify the process of colony sheath formation and the formation of related structures in race B. Our ultrastructural analysis reveals the unique nature of the colony organization of *B. braunii*, in which cells are totally enclosed by an extracellular matrix in addition to a cell wall; thin layers containing hydrocarbons form in basolateral regions and a retaining wall and the colony sheath

122 form at the cell apex.

2. Materials and methods

2.1. Strains and culture conditions

Botryococcus braunii race B was cultured in modified Chu 13 medium [34] at 22 °C on a shaker at 80 rpm. The cultures were illuminated for 13 h per day with fluorescent light at a photon flux density of $50 \mu\text{mol} \cdot \text{m}^{-2} \cdot \text{sec}^{-1}$. To induce cell division at higher rates, 1-month-old cell cultures were transferred into fresh culture medium after 1 h of illumination.

2.2. Cell collection

Cell cultures were first condensed by filtration on 10- μm nylon mesh using a magnetic filter funnel (Pall Corporation, NY, USA), and then the condensed cell culture was centrifuged using Viva-spin ultrafiltration columns (GE Healthcare Life Sciences, USA) at 700 g for 2 min to remove excess media. The cells collected on the filter were used for microscopy.

2.3. Light and fluorescence microscopy

For staining with Nile red, 1/200 volume of 1 mM Nile red solution (stock solution dissolved in dimethyl sulfoxide) was added to condensed cell culture, and the cells were soaked for 10 min. The cells were then mounted on a glass slide and lightly pressed with a coverslip. For the India ink treatment, a drop of concentrated culture was mounted on a glass slide and covered with a coverslip. Then 80% India ink solution was added to the culture from the edge of the coverslip. Cells were observed under a BX51 fluorescence microscope (Olympus, Japan) using an FITC filter set for Nile red and a bright-field for India ink.

2.4. Electron microscopy

Concentrated cell cultures of *B. braunii* cells at different developmental stages were attached to Formvar films mounted on copper wire loops 8-mm in diameter and were quickly frozen in liquid propane cooled to $-190\text{ }^{\circ}\text{C}$ by using liquid nitrogen. Frozen cells were then transferred to acetone ($-85\text{ }^{\circ}\text{C}$) that contained 3% osmium tetroxide and 0.2% uranyl acetate. After $\geq 72\text{ h}$ at $-85\text{ }^{\circ}\text{C}$, the samples were gradually warmed to $4\text{ }^{\circ}\text{C}$, treated with a new solution of 3% osmium tetroxide and 0.2% uranyl acetate in acetone for another 1 h at room temperature, washed with acetone, gradually substituted with Spurr resin (at 1, 3, 5, 10, 20, 40, 60, 80, and 90%), and finally embedded in 100% Spurr resin. Sections were stained with lead citrate for 10 min at room temperature and examined using a JEM-1230 or JEM-1011T transmission electron microscope (JEOL Ltd, Japan).

2.5. Silver hexamine stain

To detect polysaccharides under the electron microscope, 80- to 100-nm sections that were prepared as described above were mounted on nickel grids, treated with 1.5% periodic acid at $60\text{ }^{\circ}\text{C}$ for 15 min, then rinsed with distilled water for 15 min. They were then treated with silver hexamine solution (2.3% hexamethylenetetramine, 0.4% silver nitrate, 0.1% sodium tetraborate decahydrate) at $60\text{ }^{\circ}\text{C}$ for 40 min in the dark, rinsed with distilled water at room temperature for 3 min, rinsed with 2% sodium thiosulfate solution at room temperature for 3 min, and then rinsed again with distilled water. Sections were stained with 2% uranyl acetate for 6 min and then with lead citrate for 2 min.

3. Results

3.1. Colony organization

Botryococcus braunii is a colony-forming green alga with pyriform cells that are 7- to 10- μm in length and 4- to 6- μm in width. The cell apex is more rounded than the cell base (Fig. 1A). A typical *B. braunii* colony comprises several sub-colonies (daughter colonies), and cells within a sub-colony are arranged in the form of a spherical bouquet in which the rounded cell apex is directed towards the outer surface of the sub-colony (Fig. 1A, [12]). When colonies were stained with the fluorescent lipophilic dye Nile red, the cells were held together by extracellular oils that accumulated at the basolateral region of each cell (yellow in Fig. 1A). In contrast, the cell apex was not covered by extracellular oils. However, this does not mean that the cell apex is exposed directly to the growth medium, because when colonies were treated with India ink (black), we observed a zone enclosing the colony about 6 μm in thickness that repelled India ink (the white zone in Fig. 1B), presumably the colony sheath described in previous publications [12,33].

3.2. Ultrastructure of the colony sheath

We examined the ultrastructure of the colony sheath by using thin sections prepared for electron microscopy. This method revealed that the colony sheath was a fibrillar layer, about 4- to 7- μm in thickness (Fig. 2A1, double-headed arrow). Fibrils of the colony sheath extended vertically from the cell apex in colonies that had been cut longitudinally (Fig. 2A1, 2A2). This architecture is unique to *B. braunii*, as the fibrils extend not only from the cell apex but also from the outer edge of the electron-dense extracellular matrix. The latter is composed of thin layers (< 20-nm) [12], which have been shown to be composed of polymerized extracellular

hydrocarbons [33] and permeated liquid hydrocarbons [33,35]. When the colonies were sectioned near the surface of the colony sheath, it was clear that the colony sheath was not a single uniform layer, but rather was a complex of fibrillar units that originated from each cell (Fig. 2B, areas enclosed by white dashed lines).

In magnified photographs, it was clear that the fibrils of the colony sheath extended from a “retaining wall” on the cell wall at the cell apex (Fig. 2C1, c-RW), as was previously reported by Weiss et al. [33]. The 70-nm-long feet of the fibrils had a higher electron density and was wider than other parts of the fibril (Fig. 2C2, FF). These structures were previously called “protuberant tubular elements” on the retaining wall [33]. These elements were also prominent at the surface of the thin layers containing hydrocarbons (Fig. 2C1, h-RW), where an electron-transparent layer was located under the protuberant tubular elements. Fibrils of the colony sheath were not straight, but instead branched and fused with each other (Fig. 2C1, 2D), as previously shown [33].

Within a colony sheath, regions with higher electron density were often observed near the cell apexes (Fig. 2A1, 2A2, black and white arrow). They contained non-fibrillar materials that filled some of the spaces among the branched fibrils (Fig. 2D, double arrowheads). The outer surface of the colony sheath also had higher electron density than other parts of colony sheath (Fig. 2A2, 2AB, black arrow). Fig. 2E1 shows the last section that was cut parallel to the colony surface (no structure was seen in the next section), in which fibrillar networks are clearly seen. At higher magnification (Fig. 2E2), they form a mesh about 500-nm on a side that contained a finer mesh (50- to 100-nm on a side). Similar network structures were observed whenever we cut the colony sheath perpendicular to its fibrils.

3.3. Formation of the colony sheath, accompanied by shell formation and release

The colony sheath formed at the cell apex concomitant with cell division. The process is shown in Figure 3. Figure 3A is a typical cell at interphase, in which a nucleus (N) is located at the center, a cup-shaped chloroplast (ch) appears in the basolateral region, a Golgi apparatus (G) appears at the cell apex, and lipid bodies (L) lie between the nucleus and the chloroplast. These organelles generally remained in the same position within the cell throughout the cell cycle. The cytoplasm is completely surrounded by the plasma membrane and the cell wall (CW), which is a layer with a uniform thickness. The cell wall was covered with thin, electron-dense layers containing hydrocarbons at its basolateral region (*) and covered with an electron-dense retaining wall at its apex (c-RW).

When cells began mitosis, the apical region of the cell wall that lacked thin layers containing hydrocarbons bulged outward (Fig. 3B). During nuclear division, the space between the bulging cell wall and the plasma membrane at the cell apex expanded, which suggested the accumulation of amorphous materials in this space (Fig. 3C). Just after septum formation, the electron density of the amorphous materials in this space did not change (Fig. 3D). After formation of the new cell wall around each daughter cell, another type of thin layer appeared in the space (in Fig. 3E, one thin layer; in Fig. 3F, three layers; in Fig. 3G, four layers). These layers enclosed more than the apical region; they enclosed the whole daughter cell, and their formation was concomitant with the accumulation of oil droplets in the basolateral region of the cell (Fig. 3E–G, white 1,2 in E, white 1-3 in F, white 1-4 in G). The electron densities of the amorphous materials between the thin layers at the cell apex differed among the spaces (Fig. 3F), and some of these materials became fibrillar (Fig. 3H). After disruption of the mother cell's cell wall, the fibrillar materials became more prominent

in the apical extracellular space. In Figure 3H, the 1st, 2nd, and 5th thin layers remaining contact with the newly accumulated lipid layers, but the 3rd and 4th layers separate from them, the space between the daughter cell's cell wall and the 1st thin layer is occupied by amorphous materials, and the spaces more distant from the cell wall are occupied by fibrillar substances. The latter extended farther as a result of the formation of an exit space (Fig. 3I, between two arrows).

The apical parts of the thin layers with fibrils at their surface were completely separated from the apical region of the cells (Fig. 3J). These were named the “shell” by Weiss et al. [33]. Only the fibrils that had accumulated in the space between the daughter cell's cell wall and the 1st thin layer extended vertically from the cell apex and became part of the colony sheath (Fig. 3J, 3K). The regions of the colony sheath with higher electron density near the cell apexes (Fig. 2A, 2B, black and white arrow) might be fibrillar regions just after the fibrils began extending from the cell apexes. Sometimes, some of the thin layers remained and covered the cell wall until formation of the next colony sheath (Fig. 3F, 3G, 4A, 4B).

3.4. Development of thin layers and formation of shells

As described above, the thin layers seem to be key structures for the maturation of fibrillar substances to form shells at the cell apex and for the accumulation of oil droplets at the basolateral region of the cells (Fig. 3E–G). Based on observations of many sections at this stage, we found that each thin layer was continuous and that the number of thin layers ranged from one to six (usually three or more), which was the same as the number of oil droplet layers. Thus, it is likely that formation of the thin layer starts in the basolateral region of the cells, concomitant with the formation of a oil droplet layer to organize the hydrocarbon domain into discrete layers, and ends at

271 the cell apex.

272 We could follow the formation process only at the cell apex, because each thin
273 layer in the basolateral region was sandwiched between electron-dense layers of oil
274 droplets and was therefore difficult to see. Figure 4A shows the apical region of a pair
275 of daughter cells within a mother cell's cell wall, in which one thin layer (black 1) is
276 developing in the space between the cell walls of the mother and daughter cells, and
277 one oil droplet layer in the basolateral region (white 1) corresponds to this thin layer.
278 The fragments of sheets about 15- to 20-nm in thickness appear to gather to form a
279 layer within the amorphous materials that occupy this space. Note that at this early
280 stage, the amorphous materials in both the outer and inner regions of the space
281 divided by the developing thin layer are still quite similar. Electron-dense materials
282 accumulate inside the cell wall of the mother cell (***).

283 In the following stage, the two daughter cells each accumulate two layers of oil
284 droplets in the basolateral region (Fig. 4B1). In Figure 4B2, the mother cell's cell wall
285 (m-CW) is still covered by an old thin layer at the top of the cell (o-TL). Because the
286 old thin layer is not disrupted, fibrillar materials become confined between the old
287 thin layer and the old retaining wall (o-RW) on the surface of the mother cell's cell
288 wall. Between the cell walls of the mother and daughter cells, three thin layers can be
289 seen (black 1–3). Layer 1 is faint and thin. Layer 2 has electron-dense feet of the
290 fibrils on the outer surface and a layer about 100-nm thick on the inner surface. Layer
291 3 is composed of fragmented sheets approximately 25-nm thick that resemble those in
292 the region labeled with a black 1 in Figure 4A. We hypothesize that layer 1
293 corresponds to the beginning of the formation of thin layers, before a layer of oil
294 droplets has formed, and that layer 3 represents the following stage, and layer 2
295 represents the final stage, which resembles the old retaining wall in its structure;

protuberant tubular elements appear on the thin layer (Fig. 4B2; double arrowheads) and fibrillar materials form between layer 2 and layer 3.

Figure 4C shows the apex region of a daughter cell that still lies inside the mother cell's cell wall, in which four thin layers (black 1–4) are developing and in which one young oil droplet layer (white 4) and three mature oil layers (white 1–3) have accumulated in the basolateral regions. The young lipid droplet layer is sandwiched between thin layers 3 and 4, which are continuous near the oil droplets but fragmented near the cell apex (black and white arrow). Under thin layer 1, but not the other layers, a 70- to 90-nm thick layer has formed that resembles the layer under thin layer 2 in Figure 4B2. Figure 4D shows the apex region of the daughter cell after disruption of the mother cell's cell wall. Three thin layers (black 1–3) can be seen above the immature retaining wall (n-RW) that forms on the new cell wall. In addition, electron-dense granules 20- to 25-nm in diameter (black and white arrowhead) are obvious on the surface of all three thin layers, and fibrillar substances fill the spaces between the thin layers.

The development of thin layers at the cell apex is parallel to the development of a layer of oils and lipophilic biopolymers in the basolateral region, but neither type of layer developed in order of their position. Often a new thin layer (Fig. 4C, black 3) and a new oil droplet layer (Fig. 4C, white 4) formed between pre-existing thin layers (Fig. 4C, black 2 and 4). In contrast, fibril maturation from the amorphous materials between the thin layers progressed from the outer regions to the inner regions (Fig. 4B, 4C, 4D), which follows the order of separation of the mature thin layers from the cells to become shells (Fig. 3J).

The shells appear to develop in the following order: First, a faint thin layer appears (Fig. 4B2, black 1), then a fragmented sheet approximately 15- to 20-nm thick forms

(ex. Fig.4A, black 1), and electron-dense granular substances develops on the outer surface of thin layer (Fig. 4D2, black and white arrowhead). Next, a thin layer attached to the electron-dense feet of the fibrils forms on the outer surface (Fig. 4B2, black 2). In the final step, a mature thin layer that contains both the feet of fibrils and fibrils develops on the outer surface before separation from the daughter cells (Fig. 4A, o-TL).

3.5. Formation of a retaining wall

For the purposes of this paper, we will restrict our use of the term “retaining wall” to indicate a layer on the outside of a cell wall and on the surface of the extracellular thin layers containing hydrocarbons, in which fibrils of the colony sheath form and from which they extend.

Initially, granular substances 20- to 25-nm in diameter appeared on the daughter cell’s cell wall in regions that were not covered by oil droplet layers (Fig. 4A–D, black arrowhead). The amorphous substances then change into fibrils in the space between the immature retaining wall and the first thin layer (Fig. 4D2, black 1). As the granular substances disappear, concomitant with elongation of the protuberant tubular elements, they may fuse together to become an immature retaining wall by extending vertically to become fibrils of the colony sheath, probably as a result of a transition from amorphous materials (Fig. 5B, nc-RW).

Figure 5A shows the old retaining wall (oh-RW) and new retaining wall (nh-RW) on the surface of an extracellular layer containing hydrocarbons. As explained above, the old retaining wall is an electron-dense layer with protuberant tubular elements, overlying an electron-transparent layer. The electron density of the latter is similar to that of the thin layers (Fig. 5, white arrow 1–3). The new retaining wall on the oil

droplet layer (nh-RW) is composed of electron-dense tubular elements and an electron-transparent thin layer.

The edge of the mature oil layers was completely covered by a thin layer (Fig. 4B2, 4C, 5A, 5B, **). In contrast, the edges of the developing oil layers were not covered by a thin layer or were loosely covered by materials with an electron density similar to that of a thin layer (Fig. 4A, 4C, white double arrowheads).

Figure 5B shows new retaining walls on the surfaces of the cell wall (nc-RW) and of an oil droplet layer (nh-RW). The former is electron-dense whereas the latter is an electron-transparent layer with an electron-dense surface.

3.6. Polysaccharide detection

Fibrils of the colony sheath were previously reported to be polysaccharides dominated by arabinose and galactose [33]. However, this was proposed based on an analysis of only shells, not the entire colony sheath. In the present study, we confirmed the polysaccharide components of the entire colony sheath by means of silver hexamine treatment combined with electron microscopy. Several sections from one colony were separated into two groups and mounted on different copper grids; one grid was not treated (Control, Fig. 6A1, 6B1) and the other was treated with silver hexamine (Fig. 6A2, 6B2). Silver grains were detected on all fibrils of the colony sheath, but not in the spaces among the fibrils (Fig. 6A2, 6B2). This indicates that only the fibrils were composed of polysaccharides. Contrary to our expectation, the cell walls had weaker silver signals than the fibrils and they became more transparent to electrons than the non-treated cells (compare A1b to A2b, and B1 to B2 in Fig. 6). In contrast, the retaining walls and feet of the fibrils (Fig. 6A1b, 6B1, regions indicated between the [] brackets) showed no silver signals. The electron-dense

371 intercellular oil layers also showed no silver signals.

372 Figure 6B shows the apex region of the daughter cell inside the mother cell's cell
373 wall, in which four thin layers (black 1-4) are developing between the cell walls of the
374 mother and daughter cells, and in which lipid layers are developing in the basolateral
375 regions. In the amorphous materials inside the thin layers, silver grains were detected
376 (Fig. 6B2, ***).

4. Discussion

4.1. Formation of the colony sheath and shell release

Our results also clarify the process of colony sheath formation. The sheath forms at the cell apex in an area that is not covered by extracellular biopolymers containing hydrocarbons during cell division, and is accompanied by formation and release of the shell.

The formation is summarized as follows. When cells begin mitosis, the apical region of the cell wall bulges outward (Fig. 3B). During nuclear division, amorphous materials accumulate in the space between the mother cell's bulging cell wall and the plasma membrane (Fig. 3C). After the formation of new cell walls around each daughter cell, thin layers form in the amorphous materials, concomitant with the secretion and accumulation of oils in the basolateral region (Fig. 3E–G). Thin layers at the cell apex differentiate to form a structure like a retaining wall and begin to produce fibrillar substances. As the thin layers at the cell apex mature, the retaining wall begins to form on the apical cell wall and at each edge of the oil layers. After disruption of the mother cell's cell wall at the cell apex, mature thin layers attached to the fibrils separate from the cell apex moving from the outer layers to the inner ones, and become shells (Fig. 3I, 3J). In the final stages, fibrils form from a retaining wall on the daughter cell's cell wall and at the edge of oil layers, then extend vertically from the cell apex and become part of the colony sheath (Fig. 3J, 3K).

As a result of formation of the colony sheath in this manner, the colony sheath does not become a single uniform layer but rather forms a layer composed of units of the fibrillar region that originated from each cell (Fig. 2B, areas enclosed by the white dashed lines). The fibrils of the colony sheath that extend from a cell wall are replaced by new fibrils during each cell division, whereas the fibrils that extend from the

surfaces of extracellular biopolymers successively accumulate in the colony sheath, without replacement.

4.2. Retaining walls versus thin layers

The retaining walls, thin layers, and shells described in this paper are structures specific to *B. braunii*. The present results suggest that the retaining wall represents a layer that develops on the cell wall at the cell apex and on the surface of the extracellular biopolymers, and serves as the origin of the fibrils of the colony sheath. In contrast, the thin layers form in the space between the cells walls of the mother and daughter cells, and become the backbone of each cell's shell.

As the thin layers and shells are both prominent under an electron microscope, they attracted the attention of early *B. braunii* researchers, who described the thin layers as “trilaminar sheath” [8], “trilamellar structure” [36], or “external trilaminar structure by Berkaloﬀ et al. [35] and who described the retaining wall as an “internal trilaminar structure”, because these structures had two dense layers separated by a clear lumen in the samples prepared by means of chemical fixation. In contrast, we used the name “thin layer”, because we could not identify the trilamellar structures in samples of race A fixed by means of rapid freezing [32]. In the present study on race B, clear trilamellar structures were only observed in the immature thin layers (layer 1 in Fig. 4A, layer 3 in Fig. 4B, and layers 3 and 4 in Fig. 4C) and sometimes in old thin layers (Fig. 4B2; o-TL), but not in other corresponding layers (layer 2 in Fig. 4B, layer 2 in Fig. 4C, and layers 1 to 3 in Fig. 4D). Therefore, we have used the name “thin layer”. It was clear that the retaining walls in race B were not the previously described trilamellar structure.

Despite our definition of the retaining walls and thin layers, the mature retaining

wall on the apical cell wall and the mature thin layer at the cell apex are sites for the formation of similar branched fibrils festooned with tubular elements (Fig. 3J, 4A), and they develop similarly. Initially, granular substances 20- to 30-nm in diameter (black and white arrowheads in Fig. 4) appeared (compare thin layer 2 with the new retaining wall in Figure 4B2, and thin layers 1 to 3 with the new retaining wall in Fig. 4D2); subsequently, protuberant tubular elements that produce polysaccharide fibrils formed. In addition, the retaining wall on the apical cell wall festooned with colony sheath fibrils separates from the cell and might become a shell after the next cell division. Therefore, the definition of Weiss et al. [33], in which the shell was an excised segment of the retaining wall, has some justification. Currently, we have no way to know whether the shells that originated from the retaining wall and those that originated from the thin layers have the same chemical composition.

4.3. Comparison of retaining walls with different origins

Whether the retaining walls that form on the cell wall and on the extracellular biopolymers are the same structure remains unclear. The two types of retaining wall have the following similarities: First, both produce branched fibrils of the colony sheath that show a positive silver hexamine signal (Fig. 6A2b, 6B2). Second, the protuberant tubular elements on both retaining walls show a negative silver hexamine signal (Fig. 6A2b, 6B2, areas marked by the [] brackets). However, they also have the following differences: First, the retaining wall on the extracellular biopolymers originates as a thin layer at the edges of the oil layers (Fig. 5A, 5B), in contrast, the other retaining wall forms on the cell wall. Second, the tubular elements attached to the retaining wall on the cell wall are more electron-dense than those on the extracellular biopolymers (Fig. 2C1, 5B). Third, at the beginning of formation of the

retaining wall, granular structures appear at the surface of the retaining wall that forms on the cell wall (Fig. 4) but not at the surface of those that form on the extracellular biopolymers. Probably, these differences might facilitate the separation of older retaining wall with fibrils on mother cell wall as a shell from the colony sheath at the interface between these two retaining walls. Although we could not catch the process in this study, Hou et al. recently reported that glycerol treatment induced single cell release from colonies and the released single cell was come out with retaining wall and fibrillar sheath on its apical cell wall [37]. It suggests that the separation of the two retaining walls at the same boundary region is likely to occur by such treatment.

4.4. Role of the colony sheath

It has been generally accepted that the extracellular hydrocarbons produced by *B. braunii* allow the colonies to float, thereby facilitating access to the sunlight required for photosynthesis. However, the role of the colony sheath remains unclear. Weiss et al. [33] hypothesized that the sheath provides an appropriate interface with the growth medium, as is the case for the extracellular matrix of all algae, and proposed three additional possibilities: to improve flotation, provide protection from predators and pathogens, and mediate symbiotic exchanges between *B. braunii* and fibril-recognizing bacteria at the sheath boundaries.

The ultrastructure of the colony sheath revealed by the present study is basically the same as that reported by Weiss et al. [33]: both studies show that it is composed of branched polysaccharide fibrils that festoon the retaining wall on a cell wall. However, our new method revealed new information about the structure of the colony sheath: on the surface of the colony sheath, branched fibrils create meshwork structures 50- to

100-nm on a side within a larger 500-nm mesh (Fig. 2E). The colony sheath repelled India ink (Fig. 1B) even though the grains of Indian ink (50- to 80-nm in diameter) are potentially smaller than the gaps in the sheath. Therefore, non-structural materials may occupy the spaces within the fibrillar networks and may be key components that prevent penetration by larger molecules, such as Indian ink but keeping water and smaller molecules required for cell proliferation. As a recent study indicates, this structure could support high desiccation tolerance of *B. braunii* [38]. Not only for keeping the moisture, but the integrity of colony sheath can also play an important role for retaining the liquid hydrocarbons stored in the extracellular space of colony, because the liquid hydrocarbons are easily released when colonies are damaged on a glass slide pressed under a coverslip [12].

5. Conclusions

Colony organization of *B. braunii* is unique. Cells are connected by large amounts of extracellular biopolymers containing hydrocarbons at the basolateral region and enclosed by a retaining wall, from which polysaccharide fibrils extend to construct a colony sheath. The colony sheath formation occurs at the cell apex accompanied by the extracellular thin layer formation and oil accumulation at the basolateral region, and at the boundary zone the thin layers and the retaining wall are well integrated with newly secreted oils. The fibrils of the colony sheath that extend from a cell wall are replaced by new fibrils after each cell division, while fibrils in the extracellular thin layer accumulate to form the remaining part of sheath, without replacement. This manner might be a unique strategy of *B. braunii* to grow its colony through cell division with retaining the liquid hydrocarbons stored in the extracellular space of colony.

502

503 **Acknowledgments**

504 We thank Dr. Sugeru Okada for his useful discussion. This work was supported
505 by the CREST project of the Japan Science and Technology Agency.

506

6. References

1. Q. Hu, M. Sommerfeld, E. Jarvis, M. Ghirardi, M. Posewitz, M. Seibert, A. Darzins, Microalgal triacylglycerols as feedstocks for biofuel production: perspectives and advances, *Plant J.* 54 (2008) 621-639, <http://dx.doi.org/10.1111/j.1365-3113X.2008.03492.x>.
2. N.H. Tran, J.R. Bartlett, G.S. Kannangara, A.S. Milev, H. Volk, M.A. Wilson, Catalytic upgrading of biorefinery oil from microalgae, *Fuel* 89 (2010) 265-274, <http://dx.doi.org/10.1016/j.fuel.2009.08.015>.
3. A.J. Klok, P.P. Lamers, D.E. Martens, R.B. Draaisma, R.H. Wijffels, Edible oils from microalgae: insights in TAG accumulation, *Trends Biotechnol* 32(10) (2014) 521-528, <http://dx.doi.org/10.1016/j.tibtech.2014.07.004>.
4. J. Sheehan, T. Dunahay, J. Benemann, P. Roessler, A look back at the U.S. Department of Energy's Aquatic Species Program: Biodiesel from algae (1998): National Renewable Energy Laboratory, Golden, CO. <http://www.nrel.gov/biomass/pdfs/24190.pdf>.
5. Z.T. Wang, N. Ullrich, S. Joo, S. Waffenschmidt, U. Goodenough, Algal lipid bodies: stress induction, purification, and biochemical characterization in wild-type and starchless *Chlamydomonas reinhardtii*. *Eukaryot, Cell* 8 (2009) 1856-1868, <http://dx.doi.org/10.1128/EC.00272-09>. Epub 2009 Oct 30.
6. Y.M. Zhang, H. Chen, C.L. He, Q. Wang, Nitrogen starvation induced oxidative stress in an oil-producing green alga *Chlorella sorokiniana* C3, *PLoS ONE* 8(7) (2013) e69225, <http://dx.doi.org/10.1371/journal.pone.0069225>.
7. L. Davidi, A. Katz, U. Pick, Characterization of major lipid droplet proteins from *Dunaliella*, *Planta* 236 (2012) 19-33, <http://dx.doi.org/10.1007/s00425-011-1585-7>. Epub 2012 Jan 10.

- 532 8. C. Largeau, E. Casadevall, C. Berkaloff, P.Dhamelincourt, Sites of accumulation
533 and composition of hydrocarbons in *Botryococcus braunii*, *Phytochemistry* 19
534 (1980) 1043–1051, [http://dx.doi.org/10.1016/0031-9422\(80\)83054-8](http://dx.doi.org/10.1016/0031-9422(80)83054-8).
- 535 9. C. Largeau, E. Casadevall, C. Berkaloff, The biosynthesis of long-chain
536 hydrocarbons in the green alga *Botryococcus braunii*, *Phytochemistry* 19 (1980)
537 1081–1085, [http://dx.doi.org/10.1016/0031-9422\(80\)83060-3](http://dx.doi.org/10.1016/0031-9422(80)83060-3).
- 538 10. L.V. Wake, L.W. Hillen, Study of a bloom of the lipid-rich alga *Botryococcus*
539 *braunii* in the Darwin River reservoir, *Biotechnol. Bioeng.* 22 (1980) 1637–165,
540 <http://dx.doi.org/10.1002/bit.260220808>.
- 541 11. Y. Dote, S. Sawayama, S. Inoue, T. Minowa, S. Yokoyama, Recovery of liquid
542 fuel from hydrocarbon-rich microalgae by thermochemical liquefaction, *Fuel* 73
543 (1994) 1855–1857, [http://dx.doi.org/10.1016/0016-2361\(94\)90211-9](http://dx.doi.org/10.1016/0016-2361(94)90211-9).
- 544 12. R. Suzuki, N. Ito, Y. Uno, I. Nishii, S. Kagiwada, S. Okada, T. Noguchi,
545 Transformation of lipid bodies related to hydrocarbon accumulation in a green alga,
546 *Botryococcus braunii* (Race B), *PLoS ONE* 8 (12) (2013) e81626,
547 <http://dx.doi.org/10.1371/journal.pone.0081626>.
- 548 13. P. Metzger, C. Berkaloff, E. Casadevall, A. Coute, Alkadiene-and
549 botryococcene-producing races of wild strains of *Botryococcus braunii*,
550 *Phytochemistry* 24 (1985) 2305-2312,
551 [http://dx.doi.org/10.1016/S0031-9422\(00\)83032-0](http://dx.doi.org/10.1016/S0031-9422(00)83032-0).
- 552 14. P. Metzger, C.largeu, *Botryococcus braunii*: a rich source for hydrocarbons and
553 related ether lipids. *Microbiol, Biotechnol* 66 (2005) 486-496,
554 <http://dx.doi.org/10.1007/s00253-004-1779-z>.
- 555 15. P. Metzger, E. Villarreal-Rosales, E. Casadevall, A. Coute, Hydrocarbons,
556 aldehydes and triacylglycerols in some strains of the A race of the green alga

557 *Botryococcus braunii*, *Phytochemistry* 28 (1989) 2349-2353,
 558 [http://dx.doi.org/10.1016/S0031-9422\(00\)97982-2](http://dx.doi.org/10.1016/S0031-9422(00)97982-2).

559 16. P. Metzger, M. David, E. Casadevall, Biosynthesis of triterpenoid hydrocarbons in
 560 the B-race of the green alga *Botryococcus braunii*. Sites of production and nature
 561 of the methylating agent, *Phytochemistry* 26 (1986) 129-134,
 562 [http://dx.doi.org/10.1016/S0031-9422\(00\)81495-8](http://dx.doi.org/10.1016/S0031-9422(00)81495-8).

563 17. S. Okada, M. Murakami, K. Yamaguchi, Hydrocarbon composition of newly
 564 isolated strains of the green microalga *Botryococcus braunii*, *J. Appl. Phycol.* 7
 565 (1995) 555-559, <http://dx.doi.org/10.1007/BF00003942>.

566 18. P. Metzger, E. Casadevall, Lycopadiene, a tetraterpenoid hydrocarbon from new
 567 strains of the green alga *Botryococcus braunii*, *Tetrahedron Lett.* 28 (1987)
 568 3931-3934, [http://dx.doi.org/10.1016/S0040-4039\(00\)96423-2](http://dx.doi.org/10.1016/S0040-4039(00)96423-2).

569 19. A.C. Brown, B.A. Knights, Hydrocarbon content and its relationship to
 570 physiological state in the green alga *Botryococcus braunii*, *Phytochemistry* 8(3)
 571 (1969) 543-547, [http://dx.doi.org/10.1016/S0031-9422\(00\)85397-2](http://dx.doi.org/10.1016/S0031-9422(00)85397-2).

572 20. D. Matsushima, H. Jenke-Kodama, Y. Sato, Y. Fukunaga, K. Sumimoto, T.
 573 Kuzuyama, S. Matsunaga, S. Okada, The single cellular green microalga
 574 *Botryococcus braunii*, race B possesses three distinct 1-deoxy-D-xylulose
 575 5-phosphate synthases, *Plant Sci.* 185-186 (2012) 309-320,
 576 <http://dx.doi.org/10.1016/j.plantsci.2012.01.002>.

577 21. T.D. Niehaus, S. Okada, T.P. Devarenne, D.S. Watt, V. Sviripa, J. Chappel,
 578 Identification of unique mechanisms for triterpene biosynthesis in *Botryococcus*
 579 *braunii*, *Proc Natl Acad Sci USA* 108 (2011) 12260-12265,
 580 <http://dx.doi.org/10.1073/pnas.1106222108>.

- 581 22. R. Radakovits, R.E. Jinkerson, A. Darzins, M.C. Posewitz, Genetic engineering
582 of algae for enhanced biofuel production, *Eukaryotic, Cell* 9 (4) (2010) 486-501,
583 <http://dx.doi.org/10.1128/EC.00364-09>.
- 584 23. K.L. Kindle, R.A. Schnell, E. Fernandez, P.A. Lefebvre, Stabel nuclear
585 transformation of *Chlamydomonas* using the *Chlamydomonas* gene for nitrate
586 reductase, *J. Cell Biol.* 109 (1989) 2589-2601,
587 <http://dx.doi.org/10.1083/jcb.109.6.2589>.
- 588 24. H.N. Dawson, R. Burlingame, A.C. Cannonas, Stabel transformation of *Chlorella*:
589 rescue of nitrate reductase-deficient mutants with the nitrate reductase gene, *Curr.*
590 *Microbiol*, 35 (1997) 356-362, <http://dx.doi.org/10.1007/s002849900268>.
- 591 25. A. Minoda, R. Sakagami, F. Yagisawa, T. Kuroiwa, K. Tanaka, Improvement of
592 culture conditions and evidence for nuclear transformation by homologous
593 recombination in red alga, *Cyanidioscyzon merolae* 10D, *Plant Cell Physiol.* 45(6)
594 (2004) 667-671, <http://dx.doi.org/10.1093/pcp/pch087>.
- 595 26. N. Poulsen, N. Kroger, A new molecular tool for transgenic diatoms, *FEBS J.* 272
596 (2005) 3413-3423, <http://dx.doi.org/10.1111/j.1742-4658.2005.04760.x>.
- 597 27. N.A. Doetsch, M.R. Favreau, N. Kuscuoglu, M.D. Thompson, R.B. Hallick,
598 Chloroplast transformation in *Euglena gracilis*: splicing of a group III twintron
599 transcribed from a transgeneic psbK operon, *Curr. Genet.* 39 (2001) 49-60,
600 <http://dx.doi.org/10.1007/s002940000174P>.
- 601 28. R. Michael, D.J. Miller, Genetic transformation of dinoflagellates (*Amphidinium*
602 and *Symbiodinium*): expression of GUS in microalgae using heterologous
603 promoter constructs, *Plant J.* 13 (1998) 427-435,
604 <http://dx.doi.org/10.1046/j.1365-313X.1998.00040.x>.
- 605 29. T. Noguchi, Formation and decomposition of vacuoles in *Botryococcus* in relation

606 to the trans-Golgi network, *Protoplasma* 180 (1994) 29-38,
607 <http://dx.doi.org/10.1007/BF01379221>.

608 30. T. Noguchi, F. Kakami, Transformation of trans-Golgi Network during the cell
609 cycle in a green alga, *Botryococcus braunii*, *J. Plant Res.* 112 (1999) 175-186,
610 <http://dx.doi.org/10.1007/PL00013871>.

611 31. T. Noguchi, H. Watanabe, Brefeldin A effect on the *trans*-Golgi network and Golgi
612 bodies in *Botryococcus braunii* are not uniform during the cell cycle, *Protoplasma*
613 209 (1999) 193-206, <http://dx.doi.org/10.1007/BF01453448>.

614 32. M. Hirose, F. Mukaida, S. Okada, T. Noguchi, Active hydrocarbon biosynthesis
615 and accumulation in a green alga, *Botryococcus braunii* (Race A), *Eukaryotic. Cell*
616 12 (2013) 1132–1141, <http://dx.doi.org/10.1128/EC.00088-13>.

617 33. T.L. Weiss, R. Roth, C. Goodson, S. Vitha, I. Black, P. Azadi, J. Rusch, A.
618 HoIzenburg, TP. Devarenne, U. Goodenough, Colony organization in the green
619 alga *Boryococcus braunii* (race B) is specified by a complex extracellular matrix,
620 *Eukaryotic Cell* 11 (2012) 1424–1440, <http://dx.doi.org/10.1128/EC.00184-12>.

621 34. M. Grung, P. Metzger, S. Liaaen-jensen, Primary and secondary carotenoids in
622 two races of the green alga *Botryococcus braunii*, *Biochem. Syst. Ecol.* 17 (1989)
623 263-269, [http://dx.doi.org/10.1016/0305-1978\(89\)90001-X](http://dx.doi.org/10.1016/0305-1978(89)90001-X).

624 35. C. Berkaloff, B. Rousseau, A. Coute, E. Casadevall, P. Metzger, C. Chirac,
625 Variability of cell-wall structure and hydrocarbon type in different strains of
626 *Botryococcus braunii*, *J. Phycol.* 20 (1984) 377-389,
627 <http://dx.doi.org/10.1111/j.0022-3646.1984.00377.x>.

628 36. F.R. Wolf, E.R. Cox, Ultrastructure of active and resting colonies of *Botryococcus*
629 *braunii* (Chlorophyceae), *J. Phycol.* 17 (1981) 395-405,
630 <http://dx.doi.org/10.1111/j.1529-8817.1981.tb00868.x>.

- 631 37. L. Hou, H. Park, S. Okada, T. Ohama, Release of single cells from the colonial
632 oil-producing alga *Botryococcus braunii* by chemical treatments, *Protoplasma* 251
633 (2014) 191-199, <http://dx.doi.org/10.1007/s00709-013-0537-4>.
- 634 38. M. Demura, M. Ioki, M. Kawachi, N. Nakajima, M.M. Watanabe, Desiccation
635 tolerance of *Botryococcus braunii* (Trebouxiophyceae, Chlorophyta) and extreme
636 temperature tolerance of dehydrated cells, *J. Appl. Phycol.* 26 (2014) 49–53,
637 <http://dx.doi.org/10.1007/s10811-013-0059-7>.

Figure legends

Fig. 1. Fluorescent and light microscopy of colony. **A)** Nile red, fluorescent microscopy. Lipid bodies in the cytoplasm and extracellular matrix are stained with Nile red as yellow. Red color is chloroplast. **B)** Colony treated with India ink, light microscopy (bright field). The white layer repelling India ink (black) surrounds all cells of a colony. **2.** Enlargement of the rectangle area in **1.** Size bars, 20µm in panel B1, and 10 µm in panel A1, B2.

Fig. 2. Electron microscopy of colony and colony sheath. **A)** A part of colony in central section. **1.** Cells and electron-dense extracellular matrix are enclosed by colony sheath (double ended arrow). **2.** Enlargement of the rectangle area in **1** on the different section. Colony sheath is composed of fibrils vertically stretching from each cell apex and the upper edge of the electron-dense intercellular matrix. Near several cell apexes, the electron density of colony sheath is high (**1, 2**, black and white arrow). The surface of colony sheath is electron dense. **B)** Section cut upper regions of cells and colony sheath. Area enclosed by white dashed line is fibrillar unit originated from each cell. **C)** Apical region of interphase cell (longitudinal section). **1.** Fibrils of colony sheath stretch from tubular elements on the retaining wall are branched. Retaining wall on the cell wall is electron denser than that on the extracellular biopolymers containing hydrocarbons. **2.** Enlargement of the rectangle area in **1.** **D)** High electron dense region in colony sheath. Electron dense materials occupy some matrix among fibrils (double arrowheads). **E)** Cross section cut parallel to the surface of colony sheath. **2** is the enlargement of a part of **1.** The large networks of thicker fibrils contain fine fibrillar networks. CW, cell wall; c-RW, retaining wall on cell wall; FF, tubular elements on the retaining wall; h-RW, retaining wall on hydrocarbon

663 layers; PM, Plasma membrane; black arrow, electron dense colony surface; *,
664 extracellular biopolymer. Size bars, 5 μm in panels A1, B, E1, 0.5 μm in panels A2,
665 C1, D, E2, and 0.1 μm in panel C2.

666

667 **Fig. 3.** Formation of colony sheath accompanied by shell formation and release. **A)**
668 Longitudinal section of interphase cell. **B)** Cell apex at early growing stage before
669 nuclear division. Apical cell wall not covered with extracellular biopolymers
670 containing hydrocarbons, bulges out. **C)** Cell apex during nuclear division.
671 Amorphous materials accumulate between the bulging cell wall and the plasma
672 membrane. **D)** Cell apex just after septum formation and before new cell wall
673 formation. **E-G)** Thin layer formation between a mother and new cell wall. Thin layer
674 formation occurs concomitant with the accumulation of new oil droplets at the
675 basolateral region of cell. **E)** A part of longitudinal section of a pair of daughter cells
676 covered with one thin layer (1) and two oil droplet layers (white 1, 2) at basolateral
677 regions. **F)** Longitudinal section of a pair of daughter cells completely covered with
678 three thin layers and three oil droplet layers (white 1-3) at basolateral regions. The
679 spaces between thin layers at cell apex show different electron densities. **G)**
680 Tangential section of one daughter cell covered with four thin layers and four oil
681 droplet layers (white 1-4). **H)** Tangential section of daughter cell apex after formation
682 of extracellular biopolymer layers by fusion of oil droplets. The 3rd and 4th thin layers
683 separate from extracellular biopolymer layers at the left region, but not at right region.
684 The 1st, 2nd and 5th thin layers keep the connection to extracellular biopolymer layers.
685 Between cell wall and the 1st thin layer, amorphous materials occupies as like
686 previous stages and fibrillar substances appear between 1st and 5th thin layers. **I)**
687 Tangential section of daughter cell apex after separation of three thin layers at the left

border between extracellular biopolymer layers and thin layers. Fibrillar substances start to stretch from exit space (between tow arrows). **J)** Cell releasing three thin layers from cell apex. The fibrils vertically stretch from the retaining wall at apex. **K)** A pair of mature daughter cells after colony sheath formation. ch, chloroplast; CW, cell wall; c-RW, retaining wall on cell wall; G, Golgi body; h-RW, retaining wall on extracellular biopolymer layers; m-CW, mother cell wall; L, lipid body; M, mitochondrion; N, nucleus; n-CW, new daughter cell wall; S, shell; S' shell just before release; PM, Plasma membrane; V, vacuole; *, extracellular biopolymer layers. Size bars, 1 μm in panels A, F, G-K, 0.5 μm in panels B-E.

697

Fig. 4. Retaining wall formation with thin layer development at daughter cell apex. **A)** Beginning of retaining wall and thin layer formation. In amorphous materials between mother and daughter cell walls, fragments of sheets gather to make a layer (1). Granular substances appear on the new cell walls. Electron dense materials accumulate under the mother cell wall. **B)** Retaining wall and development of thin layers. **1.** A part of a pair of daughter cells accumulating two oil droplet layers at the basolateral region. **2.** Magnified photograph of rectangle area in **1**. Thin layers (black 1-3) show the developing process 1→3→2 in order. Layer 2 is similar to the old retaining wall attached with protuberant tubular elements. Amorphous substances accumulate between new cell wall and layer 2, and fibrillar materials between layer 2 and 3. Electron dense materials accumulate under the mother cell wall. **C)** Cell apex with developing 4 thin layers and oil droplet layers. Thin layers and oil layers show the developing process, black 4→3→1→2 and white 4→3→1→2, respectively. **D)** Cell apex with 3 mature thin layers and 4 oil layers. **2.** Magnified photograph of rectangle area in **1**. Granules appear on all three thin layers and on daughter cell wall,

and fibrillar substances occupy all spaces between thin layers. m-CW, mother cell wall; n-CW, new daughter cell wall; n-RW, new retaining wall; o-RW, old retaining wall; o-TL, old thin layer; oh-RW, old retaining on extracellular matrix; PM, Plasma membrane; black and white arrow, developing thin layer; black arrowhead, granule on retaining wall; black and white arrowhead, granule on thin layer; white double arrowheads, edge of hydrocarbon layer covered with immature retaining wall; black and white double arrowheads, tubular elements on thin layer; *, extracellular biopolymer layers; **, edge of extracellular biopolymer layer covered with mature retaining wall or thin layer; ***, amorphous materials under mother cell wall. Size bars, 0.5 μm .

Fig. 5. Retaining wall formation on extracellular biopolymer layers containing hydrocarbons. **A)** Old and new retaining walls. Both are composed of the electron dense layer with tubular elements and an electron transparent layer that is similar to thin layers (white arrow 1-3). The edges of mature biopolymer layers are completely covered with thin layer. **B)** New retaining wall on the oil droplet layer. Its electron density is different from that of retaining wall on the cell wall. nh-RW, new retaining wall on extracellular biopolymer layers; n-CW, new daughter cell wall; nc-RW, new retaining wall on cell wall; nh-RW, new retaining wall on hydrocarbon layer; oh-RW, old retaining wall on extracellular biopolymer layers; PM, Plasma membrane; double arrowheads, edge of extracellular biopolymer layers covered with immature retaining wall; *, extracellular biopolymer layer; **, edge of extracellular biopolymer layer covered with mature retaining wall or thin layer. Size bars, 0.5 μm .

Fig. 6. Detection of polysaccharide by silver methenamine method. **A)** **1** (non-treated)

738 and **2** (treated with silver-hexamine) are different sections from the same mature
 739 daughter cell. **1b** and **2b** are magnified photographs of rectangle area in **1a** and **1b**,
 740 respectively. Silver grains are detected on the entire fibrils of colony sheath, but not in
 741 the space among fibrils, retaining walls and fibrillar foots ([]). **B**) **1** (non-treated) and
 742 **2** (treated) are different sections from the same daughter cell within the mother cell
 743 wall. Four thin layers (black 1-4) are developing between daughter and mother cell
 744 walls, and oil droplet layers (white 2, 3) are maturing at the basolateral region. Silver
 745 grains are detected on the amorphous materials under the thin layers and mother cell
 746 wall, in addition to the fibrils of colony sheath. Retaining walls and fibrillar foots ([])
 747 are negative to silver methenamine treatment. CW, cell wall; m-CW, mother cell wall;
 748 n-CW, new daughter cell wall; black arrowhead, granules on retaining wall; black and
 749 white arrowhead, granules on thin layer; *, extracellular biopolymer; ***, amorphous
 750 materials under thin layers or mother cell wall. Size bars: 2µm in panels A1a, A2a,
 751 and 0.5 µm in panels A1b, A2b, B1, B2.

Fig.1

Fig.2

Fig.3

Fig.4

Fig.5

Fig.6