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Forced symbiosis between synechocystis spp. PCC 6803 and apo-symbiotic *Paramecium bursaria* as an experimental model for evolutionary emergence of primitive photosynthetic eukaryotes

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Single-cell green paramecia (*Paramecium bursaria*) is a swimming vehicle that carries several hundred cells of endo-symbiotic green algae. Here, a novel model for endo-symbiosis, prepared by introducing and maintaining the cells of cyanobacterium (Synechocystis spp. PCC 6803) in the apo-symbiotic cells of *P. bursaria* is described.

Single-cell *Paramecium bursaria*, or green paramecia, is often described as a swimming vehicle that carries several hundred cells of endo-symbiotic green algae that are morphologically and genetically almost identical to Chlorella species.[1] Recent bio-engineering studies have demonstrated that the high capacity for symbiotic algae inside the host cells can be replaced with various natural and artificial particles such as fluorescent and magnetic microspheres.[2,3]

This organism has attracted the attention of cell biologists, biochemists and ecologists since *P. bursaria* serves as an excellent experimental model for studying the nature of endo-symbiosis in which one species propagates inside the cells of other species under the precise control through the chemical communications between the host and symbiont cells, and knowledge on the recognition of the symbiotic partners, exchange of chemicals and regulation of metabolic processes have been documented.[4-6] It is well known that synchronization on the algal cell division is imposed by the hosting paramecia possibly through chemical communication between the partners.[7] In our recent study focusing on the impacts of the host's cell cycle and growth status on the life cycle in endo-symbiotic algae, flow-cytometric analysis has revealed that the life cycle of symbiotic algae is largely affected by the growth status of the hosting cells.[8]

Occasionally, apo-symbiotic cells of *P. bursaria* (thus lacking algae) can be found in natural water environments[9] and also in dark-grown culture of *P. bursaria*.[10] Interestingly, alga-free cell strains of *P. bursaria* can be artificially prepared by treating the stocks of green paramecia with cycloheximide[11] or some herbicides.[12,13] Some groups have shown that independently cultured apo-symbiotic host cells and ex-symbiotic algae can re-associate and re-establish the symbiotic relationship.[14,15] Due to this experimentally reproducible symbiotic nature, *P. bursaria* can be the best model for studying the mechanism (and possibly the origins) of endo-symbiosis. In our effort to elucidate the mechanism required for successful symbiosis, our recent report has described a case of symbiosis distortion leading to unregulated growth of symbiotic algae, and the significance and advantage of such material for studying the nature and origin of endo-symbiosis were discussed in reference 3.

Above studies suggest that in the evolutional time scale required for emergence of a novel photosynthetic organism, the history of symbiosis in *P. bursaria* is likely recent; thus, both the symbiont and host organisms are still on the process for developing the mechanism in which partners become highly dependent on each other. From such evolutional points of view, the symbiosis between algae and ciliate in *P. bursaria* is most likely a fruit of co-evolution between two organisms in which host species developed its tolerance to the presence of photosynthetic symbionts which behave as the source of both the sugars and photosynthesis-associated oxidative stresses.[16]

Our aim in this study was for experimentally reproducing the conditions mimicking the first contact and development of symbiosis between unicellular ciliate protozoa and photosynthetic bacteria as a novel model for studying the very early evolutional processes for the emergence of photosynthetic eukaryotes, the hypothetical ancestorial organisms of plants.[17] In fact, in

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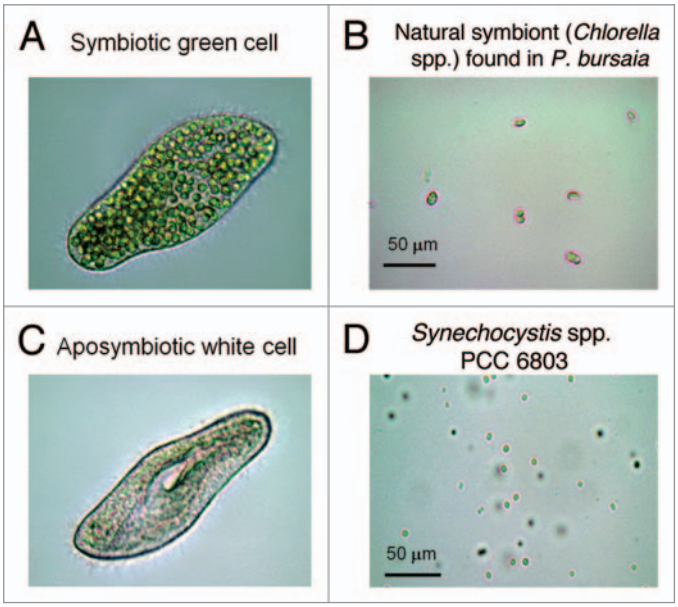

Figure 1. Light microscopic images of *P. bursaria* and its ex-endosymbiotic algae. (A) Matured cell of *P. bursaria* harboring the symbiotic green algae. (B) Ex-symbiotic algae isolated from *P. bursaria.* (C) Alga-free apo-symbiotic host cell of *P. bursaria*. (D) Cyanobacteria (Synechocystis spp. PCC 6803).

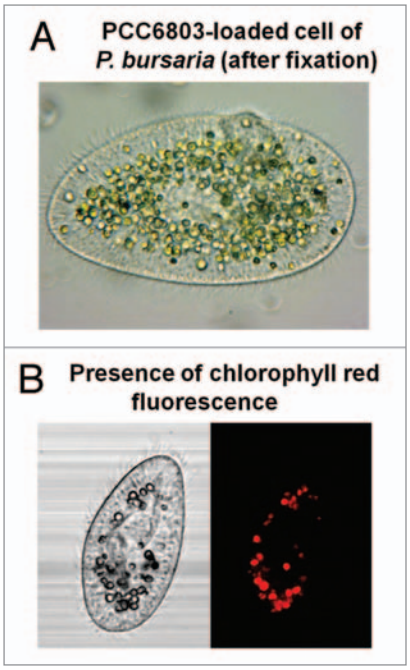

Figure 2. Microscopic images of cyanobacteria-loaded cell of *P. bursaria*. (A) Light microscopic image. (B) Laser scanning confocal microscopic images (Left: nomarski differential interference image, Right: chlorophyll fluorescence image).

our system, the preparation of apo-sympiotic *P. bursaria* (white cells) after forced algal removal from symbiotic *P. bursaria* (green cells) allows us loading of any particles of interests both biological and non-biological into the ciliate cells;[2,3] therefore the fate of experimentally loaded particles or organism is of great interest. Here, we describe a novel model endo-symbiotic complex formed between the cells of cyanobacterium (Synechocystis spp. PCC 6803) and the hosting cells derived from alga-removed *P. bursaria.*

P. bursaria strain INA-1 (**Fig. 1A**; syngen 1, shown to be mating type I as recently tested),[17] which has endosymbiotic green algae (**Fig. 1B**) was originally collected from the Ongagawa River (Kama-city, Fukuoka Pref., Japan) as described in reference 2.

Since the cell line was established after single cell isolation, all the cells in the culture were clones sharing identical genetic background. Apo-symbiotic white strain of *P. bursaria* was prepared from natural green strain (INA-1) as previously described in reference 2. These strains were maintained in the lettuce infusion inoculated with the food bacterium *Klebsiella pneumoniae* 24 h prior to the subculturing of ciliate cells, as described before in reference 8. The ciliate culture was initiated with *ca.* 10–20 cells/ml and propagated to the confluent level (over 1,000 cells/ml) under a light cycle of 12 h light and 12 h dark with *ca*. 3,500 lux (30 cm from the light source) of fluorescent natural-white light at 23°C.

The protocol of Tanaka et al.[18] was employed for preparation of apo-symbiotic white cells. Briefly, the green cells were incubated in the presence of 0.1 μM paraquat for over 24 h under light condition (with a fluorescent white lamp, 3,000 lux at least). Then, a single ciliate lacking algae was separated under a microscope and the cell line of apo-symbiotic paramecia (**Fig. 1C**) derived from this single cell was propagated in the lettuce infusion inoculated with food bacteria as described above. We found that this herbicide treatment merely enhances the excretion of algae from the ciliate but many portions of resultant ex-symbiotic algae excreted from the ciliates are still alive and capable of growing in vitro.[17]

As a model organism in order to develop an experimental model for studying the origin of photosynthetic organisms leading to evolution of plants, cyanobacteria would be the best organism since the photosynthetic apparatus in this organism is very similar to the one found in plants.[19] Among cyanobacteria as the material to be loaded to apo-symbiotic *P. bursaria* cells, Synechocystis sp. PCC 6803 (**Fig. 1D**) was chosen since it is one of the most highly studied cyanobacteria capable of growth in both autotrophical and heterotrophical manners.[20] PCC 6803 cells were propagated in BG-11 medium under a continuous light at ca. 3,000 lux with fluorescent natural-white light at 23°C. Then alga-free apo-symbiotic cells isolated and propagated after forced algal excretion, were used for the introduction of PCC 6803 cells based on the modified procedure used for re-introduction of green algae (re-greening process). Apo-symbiotic *P. bursaria* cells were incubated with suspension of PCC 6803 cells (10 μl of confluent culture of PCC 6803 into 1 ml of ciliate culture, i.e., 2,000 cells) in lettuce infusion for 30 min in an Eppendorf tube at room temperature. The resultant Synechocystis-fed re-greened cells were collected on a nylon

mesh (pore size, 10 μm), and washed out with 10 ml of fresh medium three times.

Intact live cells were used for routine observations under a stereomicroscope (SMZ645; Nikon, Tokyo, Japan; **Fig. 2A**). For obtaining the digital microscopic images with higher resolutions, the *P. bursaria* cells with and without symbionts were fixed in 3% (w/v) formaldehyde added to the culture medium and fixation was allowed at room temperature for 5 min. Confocal laser scanning microscopic red fluorescent images and differential interference contrast (DIC) images of *P. bursaria* cells were acquired using a Radiance 2100 microscope (Bio-Rad Laboratories, Hercules, CA; **Fig. 2B**). The obtained images were processed using Adobe Photoshop software.

Diagnostic polymerase chain reactions (PCR) were performed for confirming the presence of PCC 6803 cells in the one-year-old symbiotic culture; following primers and reaction condition were employed. For cyanobacteria specific detection, a set of primers was designed from the Cyanobase.[21] The PCR products were obtained from positions 1,600,000 to 1,601,783 of the chromosome. The specific primers were Forward primer 5'-GTG GTT TGG GTC AAT GTT-3' and Reverse primer 5'-CAG GGC CTT GTA AAC TTT-3'. The other standard methods for culture and DNA manipulations of cyanobacteria were described in reference 22.

Sysmex flow particle image analyzer FPIA-2100 (Sysmex Co., Kobe, Japan) was used for statistical analysis of algal cell size as previously described in reference 3. PCC 6803 cells were suspended in the sheath medium and used for direct measurement by FPIA-2100.

After addition of PCC 6803 suspension to apo-symbiotic cells of *P. bursaria*, bacterial particles were rapidly taken up by the ciliate through the oral groove. Based on microscopic observation with video, the moment that one bacterium located at the deepest end of oral groove is incorporated in cytosolic space (i.e., constriction and isolation of bacterium-containing membrane-like structure resembling the digestive vacuoles) was recorded, indicating that the ciliate takes up the PCC 6803 cells in a manner similar to take up the food bacterium. However, the fate of food bacteria and cyanobacteria drastically differed. As previously reported, the emerald green fluorescent protein (EmGFP)-labeled cells of *E. coli* can be used as a model food bacterium and its fate can be visualized by following the changes in fluorescence level and localization.[2] Based on observation of EmGFP *E. coli*-loaded apo-symbiotic ciliate, localization of bacteria in several compartments was observed; thus instead of being dispersed throughout the hosting cells, presence of food bacteria are restricted and localized within the food vacuoles (**Fig. 3B**). The time required for digestion of EmGFP-labeled cells of *E. coli* was estimated to be 30–60 min (fluorescence could not be observed after 1 h).[2]

On the other hand, the presence of PCC 6803 cells inside the host ciliate could be observed even after continuous propagation of the ciliate for over 1 year (**Fig. 3C**), thus indicative of the bacterial growth in the host cells. Initially, number of PCC 6803 cells in the host cells were limited within few particles and restricted within the localized compartments in the ciliate cells, but in the samples after continuous culturing for 1, 3, 6 and 12 months, PCC 6803 cells increased and maintained its population inside the hosting cells and their presence were shown to be dispersed throughout the cytosol of hosting cells.

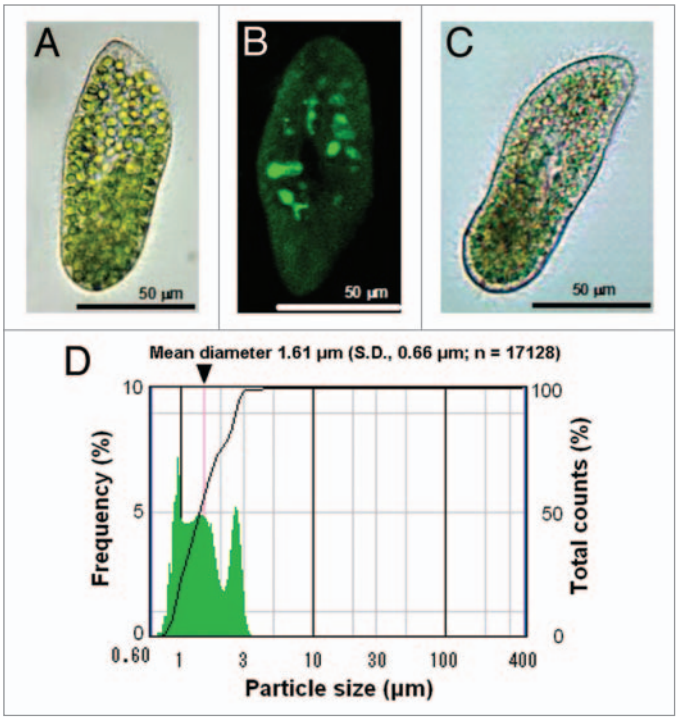

Figure 3. Continuous culturing of Synechocystis spp. PCC 6803 in the host cell. (A) Light microscopic images of wild-type *P. bursaria*. (B) Laser scanning confocal microscopic image of EmGFP-labeled *E. coli*-loaded cell of apo-symbiotic *P. bursaria*.[2] (C) Light microscopic images of Synechocystis spp. PCC 6803-loaded cell of *P. bursaria* in the one-year-old symbiotic culture. (D) Statistical analysis of Synechocystis spp. PCC 6803 cells in 1 year-old symbiotic culture using a Symex flow particle image analyzer FPIA-2100.

Irie et al.[3] tested the loading of various fluorescence-labeled microspheres, green algae and bacteria into the apo-symbiotic *P. bursaria*. According to their work, there is a tight relationship between the size of particles loaded and the mode of localization within the ciliate. Small nano-particles (sized 50–250 nm in diameter) and polystyrene micro-beads sized around 2–3 μm in diameter are likely packed as groups in localized compartments, thus some tens of particles were gathered in spherical structures (resembling the digestive vacuoles) within the ciliate cells, thus their presence was restricted. Larger micro-beads sized around 4–10 μm in diameter did not gather around each other but likely packed individually, thus showing dispersed localization throughout the cytosol of the ciliate cells.[3] While *E. coli* (sized ca. 2 μm) obeys this size-based localization pattern, PCC 6803 showed dispersed distribution similar to the localization of natural green algae (symbiotic Chlorella species), despite its small size (1.61 μm in diameter, mean size; **Fig. 3D**).

According to Tanaka et al.[18] for discussing the presence or absence of symbiotic microorganisms inside the ciliate, microscopic morphological observation is not sufficient, but instead diagnostic PCR is required. Therefore, the presence and stability of PCC 6803 cells likely propagated within the one-year-old

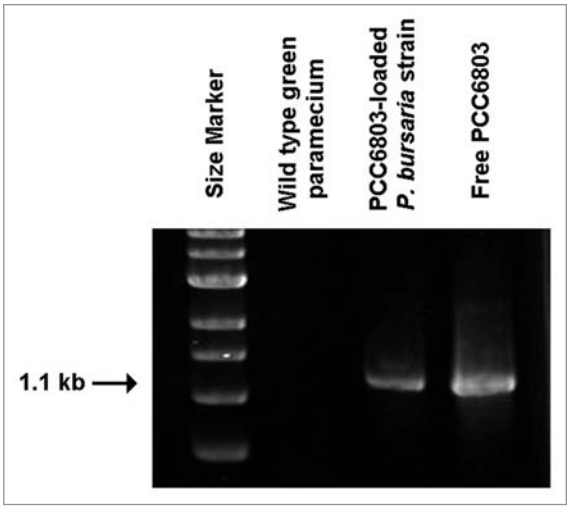

Figure 4. Diagostic polymerase chain reaction for confirming the presence of Synechocystis spp. PCC 6803 cells in host cell from one-year-old symbiotic culture.

symbiotic complex was examined by diagnostic PCR (**Fig.** 4). Presence of the genome of PCC 6803 was confirmed in the one-year-old culture of PCC 6803-loaded *P. bursaria*, supporting our microscopic observation at molecular level.

We previously proposed two possible biochemical models (models 1 and 2) explaining the co-evolution between Paramecium species and algal symbionts by focusing on the dual roles of algal photosynthesis providing the energy source and the risk of oxidative damage to the hosting cells.[16] The first model is based on the correlation between the (re)greening ability and the tolerance to oxidative stress among the paramecium species, whereas the second model is deduced through discussion on the possible evolutionary selection leading to emergence of Paramecium species tolerant against alga-derived reactive oxygen species through alga-paramecium contacts. These views could be applicable to the events required for the emergence of photosynthetic eukaryotes after incorporation of photosynthetic bacteria as primitive chloroplasts. These views should be critically tested in the future researches using our experimental model system.

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