A Green *Paramecium* Strain with Abnormal Growth of Symbiotic Algae

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Z. Naturforsch. **65c**, 681–687 (2010); received August 14, 2009/January 28, 2010

Some hundred cells of *Chlorella*-like green algae are naturally enclosed within the cytoplasm of a single cell of green paramecia (*Paramecium bursaria*). Therefore, *P. bursaria* serves as an experimental model for studying the nature of endo-symbiosis made up through chemical communication between the symbiotic partners. For studying the mechanism of symbiotic regulations, the materials showing successful symbiosis are widely used. Apart from such successful model materials, some models for symbiotic distortion would be of great interest in order to understand the nature of successful symbiosis. Here, we describe a case of unsuccessful symbiosis causing unregulated growth of algae inside the hosting ciliates. Recently, we have screened some cell lines, from the mass of *P. bursaria* cells survived after paraquat treatment. The resultant cell lines (designated as KMZ series) show novel and unusual morphological features with heavily darker green colour distinguishable from the original pale green-coloured paramecia. In this type of isolates, endo-symbiotic algae are restricted within one or two dense spherical structures located at the center of the host cells' cytoplasm. Interestingly, this isolate maintains the host cells' circadian mating response which is known as an alga-dependent behaviour in the host cells. In contrast, we discuss that KMZ lacks the host-dependent regulation of algal growth, thus the algal complex often over-grows obviously exceeding the original size of the normal hosting ciliates. Additionally, possible use of this isolate as a novel model for symbiotic cell-to-cell communication is discussed.

Key words: Algae, Ciliate, Symbiosis

Introduction

A single cell of green paramecia (*Paramecium bursaria*) is a swimming vehicle that carries several hundred cells of endo-symbiotic green algae which are morphologically and genetically almost identical to *Chlorella* species (Hoshina *et al*., 2004). Recent bioengineering studies have demonstrated that the symbiotic algae inside the host cells can be replaced with various artificial particles such as fluorescent and magnetic microspheres (Furukawa *et al.*, 2009). Then electrically driven particle transports in the capillary system can be manifested (Furukawa *et al.*, 2009) by ion channel-mediated galvanotactic migration of this swimming "micromachine" (Aonuma *et al*., 2007).

From the scientific point of view, this organism attracted the attention of 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 chemical communication 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 (Brown and Nielsen, 1974; Tanaka and Miwa, 1996; Kamako and Imamura, 2006). Kawano *et al*. (2004) have demonstrated that the symbiosis between algae and ciliates in *P. bursaria* is most likely a fruit of co-evolution between two organisms in which host species developed tolerance to the presence of photosynthetic symbionts which behave as the source of both the sugars and light-dependent oxidative stresses.

Occasionally, apo-symbiotic cells of *P. bursaria* (thus lacking algae) can be found in natural water environments (Tonooka and Watanabe, 2002) and also in dark-grown culture of *P. bursaria* (Siegel,

0939–5075/2010/1100–0681 $ 06.00

1960). Interestingly, alga-free cell strains of *P. bursaria* can be artificially prepared by treating the stocks of green paramecia with cycloheximide (Weis, 1984) or some herbicides (Reisser, 1976; Hosoya *et al*., 1995).

Some groups have shown that independently cultured apo-symbiotic host cells and ex-symbiotic algae can re-associate and re-establish the symbiotic relationship (Nishihara *et al*., 1998; Summerer *et al*., 2007). 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.

It is well known that synchronization on the algal cell division is imposed by the hosting paramecia possibly through chemical communication between the partners (Weis, 1977). In our recent study focusing on the impacts of the host's cell cycle and growth status on the life cycle of 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 (Kadono *et al*., 2004). We found that the propagation of hosting paramecia drastically governs the algal cell size, DNA contents and number of the cells (spores) within the envelope structures so-called sporangia by forcing synchronization during symbiosis.

For understanding the mechanism of symbiotic relations between different organisms, the materials showing successful symbiosis are widely used. However, apart from such successful materials, some model cases of failure in symbiosis would be of great interest in order to understand the secrets or key components required for successful symbiosis.

In the present study, we describe a case of symbiosis distortion leading to unregulated growth of symbiotic algae, as we have screened some cell lines, from the mass of *P. bursaria* cells successively exposed to paraquat treatment (resulting in excretion of algae from the hosting cells) and passive re-greening processes (some of paraquat-treated algae retaken from the media occasionally regained their growth after incubating the culture in the paraquat-free media for a certain period of time). Here we report on the microscopic observations of this novel isolate. Additionally, significance and advantage of this material for studying the nature and origin of endo-symbiosis are discussed.

Material and Methods

Organisms used and cultural conditions

P. bursaria strain INA-1 (Fig. 1A; syngen 1, mating type I) was originally collected from the Ongagawa River (Kama-city, Fukuoka Prefecture, Japan) at the sampling point INA as described previously (Furukawa *et al.*, 2009).

Since the cell line was established after single cell isolation, all the cells in the culture were clones sharing identical genetic background. An apo-symbiotic white strain of *P. bursaria* was prepared from the natural green strain (INA-1) as previously reported (Furukawa *et al.*, 2009). This strain was maintained in the lettuce infusion inoculated with the food bacterium *Klebsiella pneumoniae* 24 h prior to the subculturing of ciliate cells, as described before (Kawano *et al*., 2004). The ciliate culture was initiated with *ca.* 10–20 cells/ml and propagated to the confluent level (over 1000 cells/ml) under a light cycle of 12 h light and 12 h dark with *ca*. 3500 lux (30 cm from the light source) of fluorescent natural-white light at 23 °C.

Forced removal of green algae and re-introduction of algae

The protocol of Tanaka *et al.* (2002) 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, 3000 lux at least). Then, a single ciliate-lacking alga (Fig. 1B) was separated under a microscope, and the cell line of apo-symbiotic paramecia derived from this single cell was propagated for comparison in the lettuce infusion inoculated with food bacteria as described above.

Paraquat treatment enhances the excretion of algae from the ciliate, and resultant ex-symbiotic algae released from the ciliate are still alive and capable of gradual recovery in growth *in vitro* (Kadono *et al.*, unpublished results). In the present study, excretion of algae (de-greening) was initiated by 1 μM paraquat. Then re-entry of paraquat-treated ex-symbiotic algae into the paraquat-treated apo-symbiotic host cells and re-establishment of symbiosis were allowed during 4 weeks of incubation under a standard light regime. After these de-greening and passive re-greening processes, some re-greened cell lines

were screened and isolated for microscopic analysis.

Mating reactivity test

The mating reactivity of KMZ cells (syngen 1, mating type I) was performed according to Tanaka and Miwa (1996). Four strains namely, KN-15 (mating type I), BWK-16 (mating type II), KN-21 (mating type III), and BWK-4 (mating type IV) were used as the tester strains (all syngen 1). For comparison, INA-1 (mating type I) was also used. Since the mating reactivity is largely affected by circadian rhythm, ciliate samples maintained under so-called LD condition (light/dark regime with 8 a.m.–8 p.m. light and 8 p.m.–8 a.m. dark) were used for the test during daytime (between 1 and 3 p.m.) and nighttime (between 1 and 3 a.m.).

Microscopic analysis

Usually intact live cells were used for routine observations under a stereomicroscope (SMZ645; Nikon, Tokyo, Japan). For obtaining digital microscopic images, the *P. bursaria* cells with and without symbiotic green algae 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, USA). The obtained images were processed using Adobe Photoshop software.

Flow particle image analyzer

A Sysmex flow particle image analyzer FPIA-2100 (Sysmex Co., Kobe, Japan) was used for statistical analysis of algal cell size. Symbiotic algae were collected and suspended in the sheath medium after homogenizing the host ciliates and used for direct measurement by FPIA-2100.

Microspheres

Polystyrene microspheres (diameters: 2.28 μm, 2.88 μm, 3.87 μm, 5.60 μm, 7.32 μm, and 9.75 μm) were obtained from Bangs Laboratories, Inc. (Fishers, IN, USA). Information on the particle size was provided by the vender, and it was confirmed by flow particle image analysis using FPIA-2100 (data not shown).

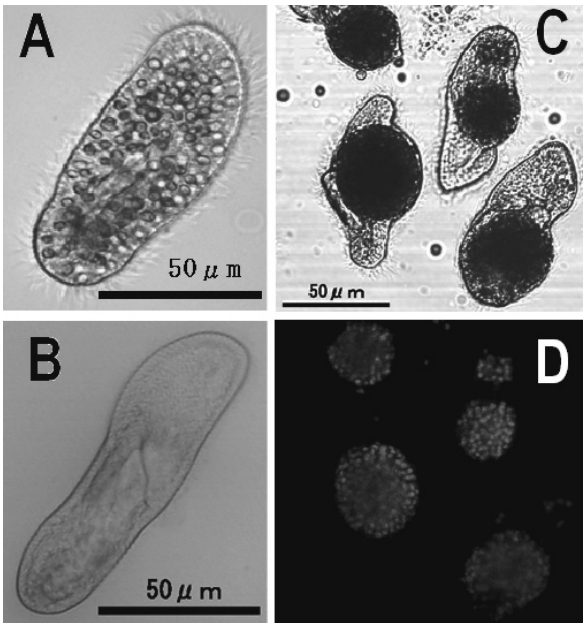

Fig. 1. Novel cell lines screened from paraquat-treated wild-type paramecia. (A) Normal green cell of wild-type *P. bursaria*. (B) Apo-symbiotic colourless cell prepared after paraquat treatment of green cells. (C) DIC and (D) red fluorescent images of KMZ cells bearing large compartments encapsulating green algae. Scale bars, 50 μm.

Results and Discussion

From the green cell line (Fig. 1A), apo-symbiotic cells can be prepared by treatment with paraquat (Fig. 1B). Among such preparations, one showed abnormal re-greening and swelling with gigantic algal complexes inside the hosting ciliate (Figs. 1C, D). The cell line (designated as KMZ) showing algal aggregating symptoms was analysed under a microscope and chlorophyll fluorescence was shown to be co-localized with the spherical structures (Fig. 1D).

Fig. 2 shows the gradual growth of algal complexes in KMZ cells. Immediately after subculturing, the presence of small algal complexes could be observed (Fig. 2A). With increasing incubation time from one week to a few months, some portions started to bear larger algal complexes (Figs. 2B–D). The number of algal complexes decreased as the size of spheres increased, suggesting that the complexes are likely merged into gigantic compartments.

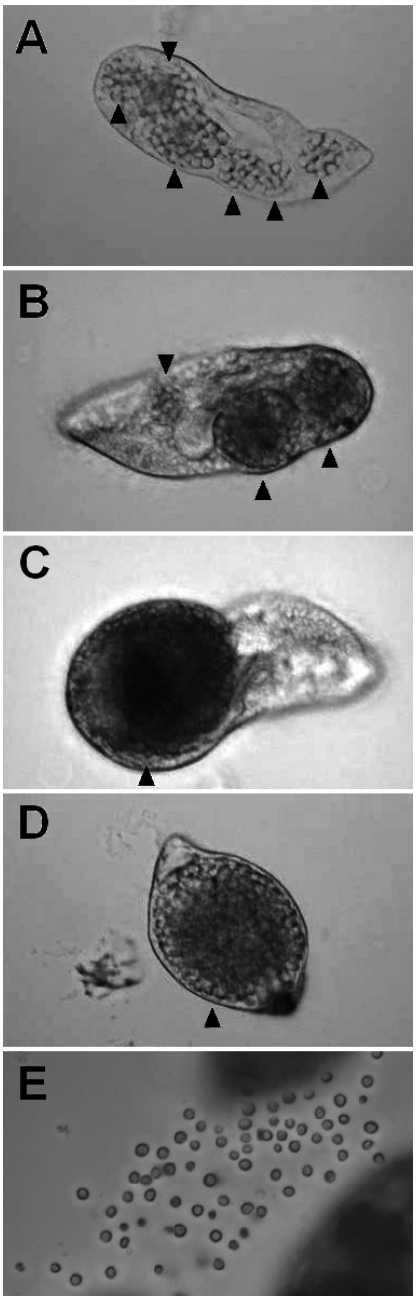

Fig. 2. Gradual growth of an algal compartment within the cells of green paramecia. Snap shots show the presence and gradual growth (swelling) of dense mass of algae over-growing in the host cells. Culture initiated by cells with several algal complexes (less dark cells) developed KMZ (algae aggregating) symptoms with time (A–D). The cells with highly swollen algal complexes (D) are readily burst out to release free algae (E). Arrowheads indicate the positions of the encapsulated algal aggregates.

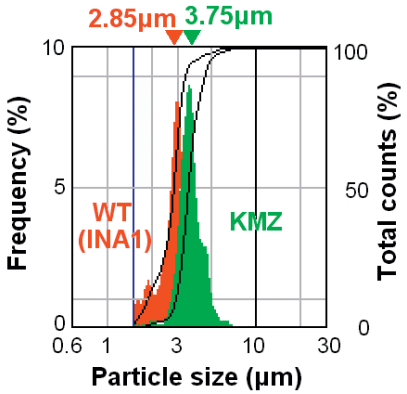

Fig. 3. Comparison of algal size obtained from wild-type (INA-1) and KMZ strains of green paramecia. After gating out the particles smaller than 1 μm, in total 398 and 621 cells were counted, respectively. Mean algal cell sizes (and standard deviation) obtained for wild-type and KMZ cells were (2.85 ± 0.67) μm and (3.75 ± 0.66) μm, respectively.

Apparently due to uncontrolled algal growth, KMZ cells over-swelled by exceeding the size of normal host cells (Figs. 2C, D), while normal green cells showed host-dependent controls of the algal cell cycle (Kadono *et al.*, 2004). Such over-swelling cells were readily punctuated and free algal particles were released (Fig. 2E). Analysis with an image analyzer showed that the sizes of algal spores from KMZ and wild-type cells were within the similar range (Fig. 3).

As the algae and particles endo-cytotically loaded to the ciliates were believed to be encapsulated in membrane structures known as perialgal or digestive vacuoles derived from oral-groove, the relationship between the size of particles and the mode of intracellular packaging was examined using polystyrene microspheres as model substrates. Microspheres sized between 2.28 and 9.75 μm were readily loaded to the apo-symbiotic cells during static incubation (Fig. 4). With finer particles (2.28 and 2.88 μm) gathered, spherical structures resembling the algal complex in the early KMZ stage were formed (Fig. 4A). Table I summarizes the typing of particle distribution inside the ciliate, according to the particle size. Our observations suggest that the particle size largely affects the modes of particle internalization but the localization of live algae could not be simply attributed to the size differences, suggesting that some unknown factors are involved in algal distribution in *P. bursaria*.

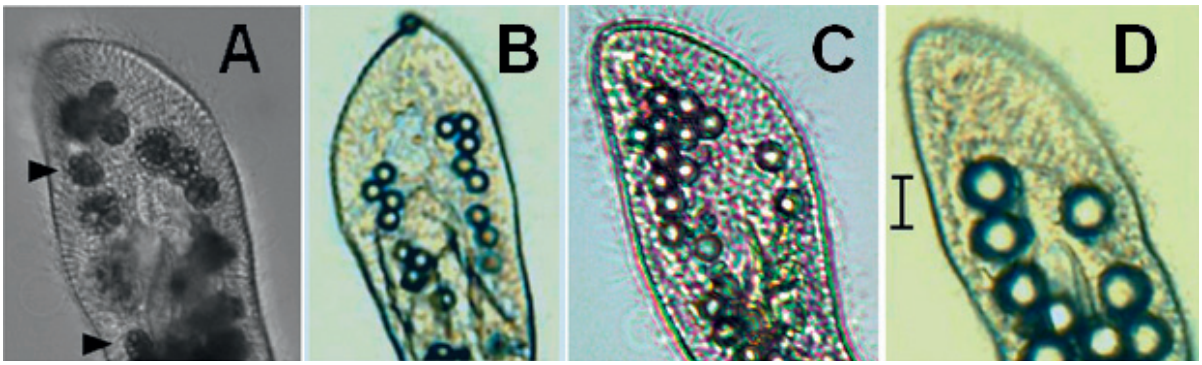

Fig. 4. Apo-symbiotic cells of *P. bursaria* loaded with artificial endocytosis substrates (microspheres). (A) 2.28-μm microsphere-loaded cell. (B) 3.87-μm microsphere-loaded cell. (C) 5.60-μm microsphere-loaded cell. (D) 9.75 μm-microsphere-loaded cell. Arrowheads indicate condensed (packaged) particles. Vertical bar, 10 μm.

Results of mating tests are summarized in Table II. During daytime, pairing of INA-1 cells and three tester strains resulted in clear cut aggregation of the cells, thus marked "+++", while KMZ cells simply formed pairs with the tester strains and kept swimming but aggregation was hardly observed, thus marked with "+". The pairs of KMZ cells and testers lasted only for *ca*. 10 min and failed to complete the further conjugation process. As the mating pair formation is one of the reliable parameters of alga-derived chemical signaling in *P. bursaria* (Tanaka and Miwa, 1996), the observed pairing between KMZ cells and testers (during the day but not during the night) is a good sign of circadian rhythmic "algae-to-host" signaling in KMZ cells.

A previous flow-cytometric study revealed that the life cycle, cell size, DNA contents and number of the algal cells (spores) within a single sporangia, and overall population of algae within the host cells are strictly controlled by the growth status of the hosting cells (Kadono *et al*., 2004; see model in Fig. 5A). In contrast, the dense algal communities encapsulated in the intracellular compartments in KMZ cells apparently failed in "host-to-algae" signaling to cope with the host cells in order to synchronize their cell cycles. Therefore, the algal complexes often over-grow obviously exceeding the original size of the normal hosting ciliates (Fig. 5B).

Our microscopic observations on the mode of KMZ cells' propagation are summarized in

Table I. Effect of particle size on the modes of endo-cytotic particle packaging within the cells of apo-symbiotic *Paramecium bursaria.*

Type of particle	Particle diameter	Mode of packaging within ciliate cells
Indian ink	50–250 nm	Restricted within digestive vacuoles[a]
Bacteria (*E. coli*)	*ca.* 2 μm	Restricted within digestive vacuoles[a]
Polystyrene microspheres	2.28 μm	Gathered in small compartments[b]
Polystyrene microspheres	2.88 μm	Gathered in small compartments[a]
Symbiotic algae in INA-1	2.85 μm	Individually (packed) and dispersed[c]
Symbiotic algae in KMZ	3.75 μm	Multiple cells packed in large spherical compartments[c]
Polystyrene microspheres	3.87 μm	Mostly dispersed, partly gathered[b]
Polystyrene microspheres	5.60 μm	Individually (packed) and dispersed[b]
Polystyrene microspheres	7.32 μm	Individually (packed) and dispersed[d]
Polystyrene microspheres	9.75 μm	Individually (packed) and dispersed[b]

[a] Typical microscopic images are available in Furukawa *et al*. (2009).
[b] See Figs. 5A–D.
[c] For typical images, see Figs. 1 and 2.
[d] Original data, images not shown.

Table II. Mating reactivity of INA-1 and KMZ cells.

Tester used (mating type)	Pairing with INA-1 (mating type I)		Pairing with KMZ (mating type I)	
	1–3 a.m.	1–3 p.m.	1–3 a.m.	1–3 p.m.
KN-15 (I)	–	–	–	–
BWK-16 (II)	–	+++	–	+
KN-21 (III)	–	+++	–	+
BWK-4 (IV)	–	+++	–	+

Symbols: (–), lack of response; (+), pairs formed; (+++), pairs formed and cell aggregation observed.

Figs. 5B–F. Due to over-swelling and burst of the cells, the rate of propagation is limited, but occasional excretion of algae from the host cells helps to avoid the burst of the cells (Fig. 5B). KMZ cells often struggle to form the constriction during cell division (Fig. 5C). Upon successful division, two unevenly greened daughter cells are likely formed (Fig. 5D). However, the culture is not yet dominated by colourless cells, even though this type of uneven cell division has been repeated for a number of generations in recent 2 years. Therefore, we propose an alternative model in which the size of algal complexes in two daughter cells is adjusted by the growth of small algal complexes in the post-division phase (Fig. 5E). In addition, uptake of ex-symbiotic algae by daughter cells also explains the dominancy of green cells in the culture (Fig. 5F).

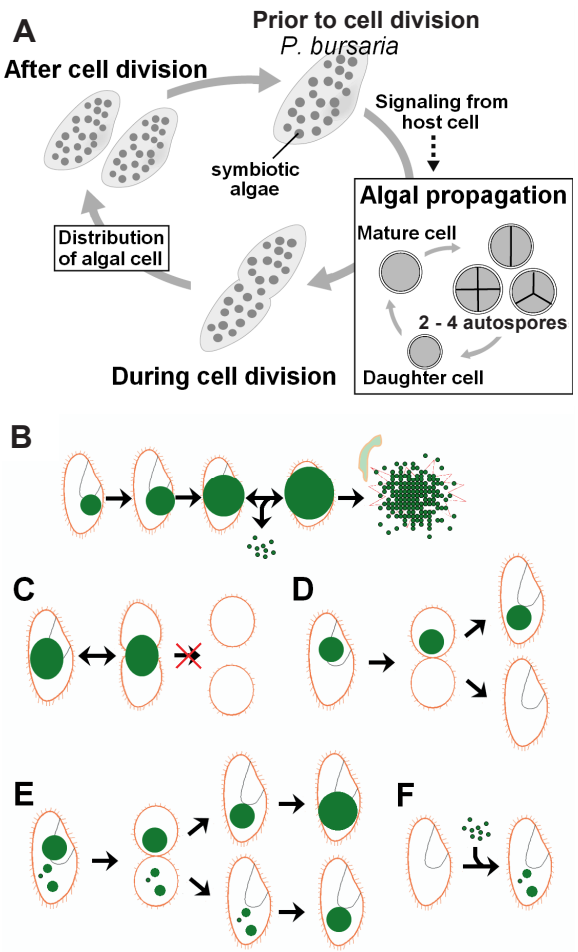

Fig. 5. Hypothetical models of KMZ cell propagation. (A) The relationship between host cell division and algal cell division in wild-type green paramecia. Earlier studies support that the algal cell division is stimulated prior to or during the division of the host cells, thus leading to an increase in sporangia with 1–4 autospores in the *Paramecium* cells. After host cell division, symbiotic algae are distributed almost equally to two daughter cells. The size of sporangia and algal population never exceed the capacity of *Paramecium* host cells. (B) Uncontrollable growth of algal complexes (macrosphere) in KMZ cells leading to the burst of the hosting paramecia. (C) Inhibition of cell division (constrict formation) by the presence of a large algal complex in KMZ cells. (D) Uneven distribution of moderate-sized single algal macrosphere during cell division of a KMZ cell results in production of both a green KMZ-type cell and a colourless apo-symbiotic cell. (E) Even if algal complexes in a KMZ cell were unevenly distributed during the host cell division, less greener daughter cells (with the seeds for algal macrosphere development) gradually turned to greener, algal compartment-bearing host cells. (F) Free algal particles released from KMZ cells (see B) can be re-loaded into the apo-symbiotic alga-free paramecia. The illustration in (A) was adopted and modified from Kadono *et al.* (2004).

Acknowledgement

This work was supported in part by Knowledge Cluster Initiative from Ministry of Education, Culture, Sports, Science and Technology (MEXT), Japan. Tester strains were a generous gift from Prof. K. Kosaka (Hiroshima University, Japan).

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