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Establishment of a Method to Culture a Washed and Cloned Green Paramecium *(Paramecium bursaria)*

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Abstract: The green paramecium, *Paramecium bursaria*, widely distributed in freshwater habitats around the world, has hundreds of symbiotic green algae in its cytoplasm. *P. bursaria* is classified as a paramecium, a species of ciliate. Symbiotic algae cannot exist inside paramecia other than *P. bursaria*, like as *P. tetraurelia* or *P. caudatum*. Much interest has been accumulating in elucidating the symbiotic mechanism of symbiotic algae that can exist only inside *P. bursaria*. However, the basic properties related to *P. bursaria* and symbiotic algae have not yet been fully elucidated. Are the species of symbiotic algae in *P. bursaria* uniform or diverse? Are the symbiotic microorganisms in *P. bursaria* symbiotic algae only? Does each individual *P. bursaria* show physiologically similar properties regarding the rate and frequency of cell division and also in terms of longevity? Actually, many things described above still remain unanswered. In this study, after isolating, washing and cloning *P. bursaria*, the rate of proliferation was measured for individual cells. Although each cloned strain should have the same genetic background, we obtained interesting results showing that the proliferation rates were significantly varied among the strains.
Keywords: green paramecium, *Paramecium bursaria*, symbiotic algae, symbiosis, cloning

Introduction

Paramecium bursaria (P. bursaria) harbors hundreds of symbiotic algae in its cytoplasm.

It has been reported that "algae-free" *P. bursaria* (white *P. bursaria*) has been produced in the laboratory[1, 2] and also that such paramecia actually exist in the field[3]. However, in the case of white *P. bursaria*, it has not been confirmed that the symbiotic algae are completely removed from the cell body.

Papers published so far regarded the observation that green algae could not be seen in the cell body of *P. bursaria* under an optical microscope or that autofluorescence emitted by symbiotic algae was not detected under a fluorescent microscope, as proof of removal of symbiotic algae[1, 2, 4]. These criteria suggest that the chlorophyll pigment of the symbiotic algae does not exist in the cell body of *P. bursaria* but are not a direct proof that the symbiotic algae have been completely removed. For a long time, experiments of re-symbiosis of symbiotic algae, in which symbiotic algae were administered to the white *P. bursaria* and the symbiotic algae were taken up into the cell body, were often carried out. However, it was impossible to determine whether the symbiotic algae were newly taken up from the outside and proliferated in the cell body, or the original symbiotic algae remaining in the white *P. bursaria* were stimulated to proliferate by the additional symbiotic algae from the outside. Until the complete removal of symbiotic algae is proved, it is difficult to regard the uptake of symbiotic algae into white *P. bursaria* as the establishment of re-symbiosis.

It has been revealed that the ribulose-1,5-bispho-

sphate carboxylase small subunit (rbcS) encoding gene present in photosynthetic plant cells was no longer detected in the white *P. bursaria*[5], which were generated by the treatment with a photosynthesis inhibitor paraquat and in which the autofluorescence of symbiotic algae was no longer detected. Thus, we were one step closer to the proof of algal removal from *P. bursaria*.

Are the hundreds of symbiotic algae in *P. bursaria* the same species in all individuals? Furthermore, are there the same species or the same proportion of symbiotic algae in *P. bursaria* found all over the world? The basic matters regarding the characteristics of such individual or regional differences in symbiotic algae remain unclear. Under these circumstances, there is no guarantee that the results obtained with *P. bursaria* isolated in one area will be consistent with those using *P. bursaria* isolated in another area.

In order to further develop symbiosis research on *P. bursaria*, it is an urgent task to make the quality of *P. bursaria* stabilize, which has so far not been uniform. For the first step to supply *P. bursaria* having stabilized quality, it is important to clarify what kind of property difference is observed in each individually cloned *P. bursaria* that should have the same genetic background. In this study, for each individual in a *P. bursaria* clone obtained using a unified method, its proliferation was observed over time.

Materials and Methods

1. Preparation of lettuce medium

Lettuce medium was used for the culture of *P. bursaria* [6]. The preparation of lettuce medium is as follows: After washing leaves of the lettuce, they were soaked in boiling tap water for 1 minute. After boiling, they were soaked in tap water at room temperature, spread on filter paper and dried in a dry heat sterilizer at 75℃ for 3 hours and further at 65℃ for 3.5 hours. The dried lettuce leaves were crushed to an appropriate size and stored in a jar inside a desiccator at room temperature. 1.5 gram of the stocked lettuce leaf was added to 3 L of deionized water in a 5 L Erlenmeyer flask, and extraction was performed at 100℃ for 5 minutes. After the extract cooled, it was filtered using filter paper (Advantec, No. 2, 50 cm) to remove tiny pieces of lettuce leaves. The lettuce medium thus obtained was dispensed into wide-mouth medium bottles, autoclaved (121℃ , 20 minutes), and stored at 4℃ until use.

2. Isolation and cloning of *P. bursaria*

A strain KUNY-2 has been originally established in 2016 and stocked in the bacterized lettuce medium[7]. In 2017, the strain was further cloned and washed with the sterilized lettuce medium, as described in Fig.1. This strain was designated as KUHH-4 and has been stocked in the sterilized lettuce medium under no-feed condition until use. One single *P. bursaria* was isolated using a micropipette from the stock (KUHH-4), washed with sterilized lettuce medium as follows, and then cultured in the sterilized lettuce medium. Four drops of the lettuce medium were placed on a slide glass, and isolated individuals of *P. bursaria* were allowed to swim in the first drop, and transferred to the second drop. This procedure was further repeated two times (Fig. 1). By this operation, we confirmed the isolation of one individual (cloning) and removal of microorganisms existing in the original culture media (washing). Then, 3 mL of sterilized lettuce medium was placed into each well of a 6-well plate (TPP 92006, flat bottom), and one individual *P. bursaria*, treated by the above-mentioned isolation and washing four times, was put into each well. The cells were cultured in an incubator (Biotron, LH-411S) at 23℃ under light irradiation (12 hours light, 12 hours dark). Two

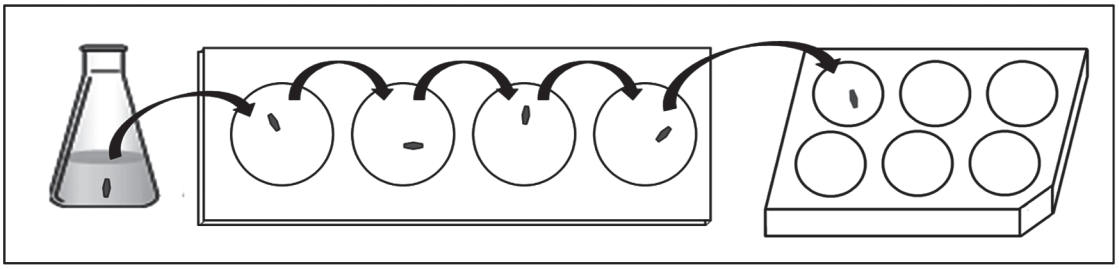

Fig. 1. Overview of cleaning and cloning methods for *P. bursaria*.

weeks after the start of culture, strains that grew to about 20-30 cells/well (7-10 cells/mL in density) were selected and placed in a 100 mL sterilized lettuce medium (200 mL Erlenmeyer flask) and growth was observed. About 40-60 days after the start of the culture, the cells grew to a density of 1,000-1,400 cells/mL and were stored in the incubator for a long period of time in that state (the steady phase of growth).

Results and Discussion

As mentioned above, for *P. bursaria* collected either in Japan or overseas, it is unclear whether the same type of symbiotic algae exist in all individuals or whether the symbiotic algae (about 400 cells) inside one individual are of the same species.

For multiple *P. bursaria* individuals (clones) obtained by the above method, growth was started from one individual in each well (3 mL sterilized lettuce medium) of a 6-well plate, and the number of cells in each well was observed over time. The growth densities around 73 days and 98 days from the start of growth are shown in (a) and (b) of Table 1 as growth index. The growth index was designated as the number of *P. bursaria* cells in one well at around 73 days or 98 days of growth divided by the initial number of cells in one well (1 cell), respectively. From Fig. 2, which shows the average values of 13 data in (a) and 11 data in (b), it was found that *P. bursaria* reached the stationary phase of growth about 2 months after the start of culture. In addition, as shown in Table 1, it was found that there are some strains, even arrived at the stationary phase, in which the cell density suddenly decreases. By repeating the cloning, the individuals, whose proliferation was measured, should have the same genetic background. However, as is clear from Table 1 and Fig. 2, the proliferation rate considerably varied among individual clones. It is natural to think that these differences in proliferation ability are derived from symbiotic algae and/or other microorganisms existing in the cell body of *P. bursaria*. Specifically, it seems highly possible that the species of symbiotic algae in each individual *P. bursaria* are not uniform but diverse in the first place and that the extent of diversity is different for each *P. bursaria* individual. In addition, it is also possible that symbiotic microorganisms other than symbiotic algae exist in *P. bursaria*[8] and they induce the proliferation diversity of the host.

In the present study, it is revealed that there is a large difference in the proliferation rate among individuals of *P. bursaria*, which are considered to have the same genetic background. From this, it is newly indicated that the growth of the host may be controlled by symbiotic algae and other symbiotic microorganisms. It is an urgent task to clarify what are the specific factors controlling the growth of *P. bursaria*.

Table 1. The growth indices at (a) around 73 days and (b) around 98 days after the start of culture. I stands for Iwanaga (2 samples). O, Ominato (4); K, Komiya (1); S, Suzuki (2); M, Matsushima (2); Y, Yokoyama (2).

(a)

days	#		growth index
72	I	1	54
		2	537
73	O	1	1446
		2	308
		3	847
		4	3170
74	K	1	989
73	S	1	8
		2	797
73	M	1	312
		2	742
74	Y	1	1301
		2	814

(b)

days	#		growth index
100	I	1	150
		2	667
98	O	2	1287
		3	3766
		4	1791
98	S	1	11
		2	1202
99	M	1	136
		2	1941
92	Y	1	854
		2	792

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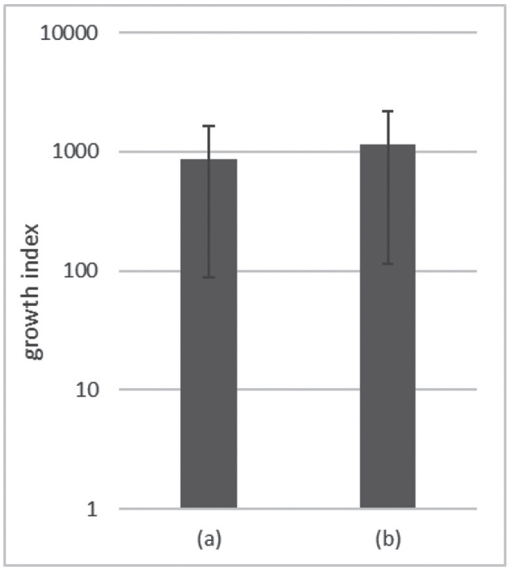

Fig. 2. The bars represent the average values growth indices shown in (a) and (b) of Table 1, respectively (error bars: standard deviations).

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