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Characterization of Symbiotic Algae-free Strains of *Paramecium bursaria* Produced by the Herbicide Paraquat

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ABSTRACT

As is well known, *Paramecium bursaria* has many endosymbiotic algae in its cytoplasm. We already reported that these endosymbiotic algae can be removed from the cytoplasm by using the herbicide paraquat and symbiotic algae-free paramecia were prepared (Hosoya et al. 1995). In this study, we examined the characteristics of symbiotic algae-free paramecia obtained by paraquat treatment. Symbiotic algae-free paramecia had a shorter log phase than those with symbiotic algae, and showed lower cell density at the stationary phase. Additionally, they had the ability to form stable symbiotic relationships with symbiotic algae. Symbiotic algae obtained from *P. bursaria* in log phase showed a higher infection rate than those from *P. bursaria* in stationary phase. These results suggest that growth phase of the host influences the reinfection ability of their interior symbiotic algae.

INTRODUCTION

The green paramecium *P. bursaria* is known to have many symbiotic algae in its cytoplasm. It is known that the green paramecia can be deprived of their symbiotic algae by several methods and symbiotic algae-free strains are established (Jennings 1938; Wichterman 1943, 1947, 1948; Siegel 1960; Karakashian 1963, 1975; Pado 1965; Weis 1969, 1974; Reisser 1976; Iwatsuki and Naitoh 1981). Unfortunately, although it is necessary to determine whether the algae-free paramecia are normal except for the absence of their symbiotic algae, no identification of physiological damage has been attempted in these cases.

We found that paraquat was most effective for producing algae-free cell lines of *P. bursaria* as previously reported (Hosoya et al. 1995). These cell lines can proliferate normally under conventional culture conditions supplemented with bacteria as food.

When these algae-free paramecia were crossed with different mature mating type strains, they performed normal sexual events. Moreover, we evaluated whether these algae-free paramecia, when they fed symbiotic algae derived from green paramecia, had the ability to maintain them in their cytoplasm. How the growth phase of both algae-free paramecia and the donor green paramecia influence the retrieval of the symbiotic relationship was also examined. We also discuss about the characteristics of the obtained algae-free paramecia.

MATERIALS AND METHODS

Culture conditions

Three strains of *Paramecium bursaria* syngen 1 (OK-312, mating type I; OKw-312, I; OZ-3, III) were used in this work. The strain OKw-312, an algae-free cell line, was obtained from strain OK-312 treated with the herbicide paraquat (Hosoya et al. 1995). These strains were cultured in lettuce infusion containing bacteria *Klebsiella pneumoniae*, and under a light/dark cycle (12 hrs light / 12 hrs dark at around 1000 Lux of natural-white fluorescent light) at 23 ± 1°C after the method of Hosoya et al. (1995). In each experiment, 200 cells from the stationary phase of stock culture were initially transferred to a petri dish (6 cm in diameter) containing 10 ml of newly prepared bacterized lettuce infusion and then were maintained under the same conditions as the stock cultures.

Measurement of cell size

Green and algae-free paramecia were separately cultured in different petri dishes containing 10 ml newly prepared bacterized lettuce infusion at initial cell density of 20 cells/ml under the same conditions as the stock cultures. After 6 and 30 days in culture, about 130 cells from each culture were transferred to drops of lettuce infusion, containing 2 % (w/v) methylcellulose to suppress their movement on glass slides and were covered by cover slips. The length of the longitudinal cell axis of 102 cells in each sample was measured with an ocular micrometer under the light microscope. The data were presented as a mean with standard deviation.

Growth curves

Green, algae-free and reinfected paramecia were used in this work. Each of them obtained from stationary phase was harvested by hand-operating centrifugation and washed twice with fresh lettuce infusion. In each culture, 200

cells were then transferred to a petri dish containing 10 ml of newly prepared bacterized lettuce infusion and cultured under the same conditions as the stock cultures. Cell density of each culture was determined by thoroughly stirring to equally distribute the cells, and then 0.1 ml of cell suspension was transferred to a depression slide. These cells were counted and transferred back from the depression slide to the original petri dish by pipetting under a stereoscopic microscope. The remaining culture medium of the depression slide was also returned to the petri dish. This procedure was repeated 5 times and the mean cell density of the culture was calculated.

Reinfection

Green paramecia (OK-312) from each growth phase were harvested by hand-operating centrifugation, and washed 3 times in fresh lettuce infusion. They were then transferred to a 1.5 ml plastic tube including 1ml lettuce infusion at 2,500 cells/ml and sonicated for 30 s (Branson Sonifier Model 450, output 2). Under these conditions, green paramecia were completely disrupted, but symbiotic algae were not affected in appearance. Then, algal density of the suspension was determined using a Toma's hematocytometer. Subsequently, 100 algae-free paramecia were added to the algal suspension. At this time, cell density of algae was adjusted to approximately 1.0×10^4 algae/paramecium, and incubated for 24 hrs under the same conditions as the stock cultures. Each paramecium was then washed 3 times with fresh lettuce infusion to remove freely suspending algae in the medium. These paramecia were individually transferred to bacterized lettuce infusion drops (15 μl / drop), which were placed on the bottom of a disposable plastic petri dish (9 cm in diameter), as each drop contains one paramecium. Petri dish was then carefully turned upside down and placed on the cover of the dish covered with wet filter paper. As a result, all drops including paramecium were hanged down from the bottom of petri dish (hanging-drop). Under these experimental conditions, the algae released from the cytoproct of the paramecia were sometimes observed. When such algae were taken in the paramecia again, the symbiotic relationship between them seemed to be re-established in appearance. To prevent such a situation, each of the paramecia in the drops was washed and transferred again to newly prepared drops at about 24 hrs and 48 hrs after start of hanging-drop culture. Algae-free paramecia were also fed free-living Chlorella that does not reinfect paramecia. Free-living Chlorella, once taken up by the algae-free paramecia, was digested or excreted: within about 3 days, none was observed in the paramecia cells (our unpublished data). When green paramecia are observed under a fluorescence microscope, only the symbiotic algae are known to appear red (Hosoya et al. 1995). In this study, after five days in a hanging-drop

culture, the presence of symbiotic algae in the cytoplasm of the paramecia was examined by observation using a fluorescence microscope (Nikon, Optiphot BFD2) to determine the reinfection rate. Paramecia, when cultured in hanging-drops at 1 cell / drop, reach 2 to 8 cells / drop in 5 days. Thus, if at least a single paramecium in a single drop had symbiotic algae in its cytoplasm, the cell line was considered to be reinfected. The reinfection rate was expressed as the ratio of the number of reinfected cell lines to the total number of examined cell lines.

RESULTS AND DISCUSSION

Trace of symbiotic algae in the cytoplasm of algae-free paramecia

OKw-312 is derived from paraquat-treated paramecia of stock OK-312. The mating type of these algae-free paramecia was type I. Hence, when it was mixed with green paramecia (OZ-3, mating type III), agglutination of the cells was observed, and the conjugants were formed several hours later. When these conjugating pairs were observed under a fluorescence microscope, symbiotic algae in the green paramecia were clearly observed as red images, because algal chlorophyll fluoresces red. However, no fluorescence was detected in algae-free paramecia (Fig. 1). We have reported that algae-free paramecia obtained by paraquat treatment had the ability of conjugation with green paramecia, and that the viability of their exconjugant clones was more than 90% (Hosoya et al. 1995).

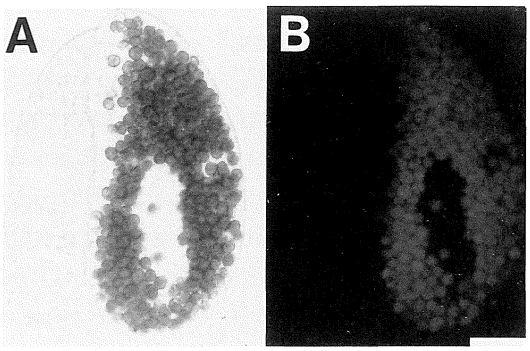

Fig. 1 Nomarski differential interference contrast (A) and the fluorescence images (B) of a conjugating pair of algae-free (OKw-312) and green (OZ-3) paramecia. Here, the algal chlorophyll fluoresces red but no fluorescence was detected in the cell treated with paraquat. Scale bar = 20 μm.

Green and algae-free paramecia used in conjugation experiment were both at the stationary phase, and yet the algae-free paramecia seemed to be relatively smaller than green paramecia. Therefore, we compared algae-free (OKw-312) and green (OK-312) paramecia with respect to their cell sizes. After 6 days in

culture, the mean cell length of OKw-312 or OK-312 was 108.7 ± 12.5 μm or 109.3 ± 10.0 μm, respectively. Little difference in them was noticed ($P \geq 0.5$; t-test). However, after 30 days in culture, OKw-312 had become shorter in the mean cell length (91.0 ± 8.6 μm) and more cells of smaller size were detected than in OK-312 (111.5 ± 7.2 μm) ($P \leq 0.01$; t-test).

Growth curve of algae-free paramecia

To determine the characteristic changes caused by the presence or absence of symbiotic algae, the growth curves of algae-free (OKw-312) and green (OK-312) paramecia were compared (Fig. 2). Although growth rate of OK-312 tended to be faster than that of OKw-312, both paramecia proliferated exponentially for the first 7 days in culture. Thereafter, OKw-312 reached the stationary phase and entered the decline phase after 20 days in culture. In contrast, no decrease in cell density of OK-312 was observed for at least 40 days in culture. OKw-312 and OK-312 had different cell densities at stationary phase, with that of OKw-312 being lower. However, reinfected paramecia (named OKr-312), which were infected by symbiotic algae obtained from OK-312, showed almost the same growth curve as in OK-312. Since it is well known that symbiotic algae release their photosynthetic product of sugar (Muscatine et al. 1967; Brown and Nielsen 1974), OK-312 and OKr-312 appear to be supplied nutrients derived from the symbiotic algae as well as the bacterial food supply. On the other hand, OKw-312 is able to use only the bacterial food supply for its nutrition. Therefore, the difference of the growth curve between green and algae-free paramecia might be dependent upon the lack of nutritions derived from the symbiotic algae. These findings can be interpreted as the result of the influence of symbiotic algae removal on the cell division of green paramecia, instead of the influence of paraquat treatment.

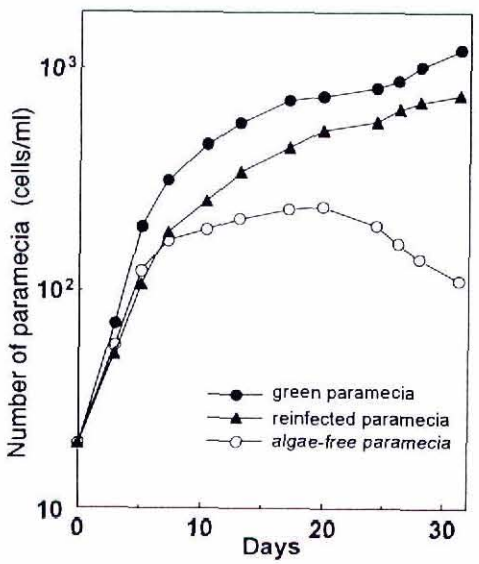

Fig. 2 The growth curve of green (●), algae-free (○) and reinfected (▲) paramecia. Results were expressed as the mean of 5 cell lines.

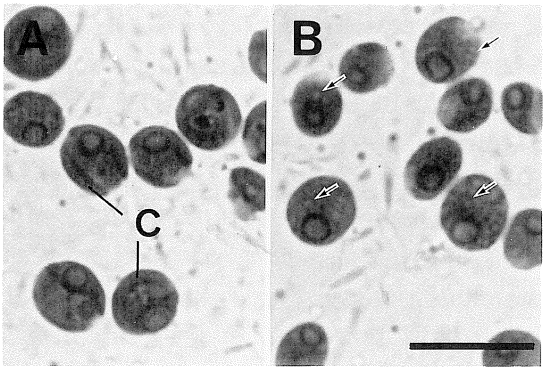

Fig. 3 Light micrograph of symbiotic algae obtained by squashing the green paramecia (OK-312) on a slide glass. (A) Log phase, after 10 days in culture. (B) Stationary phase, after 30 days in culture. Vesicles were detected in symbiotic algae at the stationary phase of green paramecia (arrows). C: chloroplast. Scale bar = 10 μm.

Furthermore, we found that the form of symbiotic algae in green paramecia changed with the progression of the host growth phase. Pyrenoid and girdle type chloroplasts were clearly observed in the interior of symbiotic algae in log phase green paramecia after 10 days in culture (Fig. 3A). On the other hand, after 30 days in culture, symbiotic algae in stationary phase green paramecia had pyrenoids, but chloroplasts were not clearly observed and many vesicles were detected in the interior (Fig. 3B, arrows). Moreover, various vesicles were also present in the cytoplasm of green paramecia. Effect of the growth phase of green and algae-free paramecia on the establishment of symbiosis

Table 1. Effect of growth phase on infection rate (%) of symbiotic algae to algae-free paramecia

Origin of algae (green paramecia) \ Algae-free paramecia		Growth phase of OKw-312	
		Log *1	Stationary *2
Growth phase of OK-312	Log *3	66.7 ± 4.8	48.0 ± 6.8
	Stationary *4	45.2 ± 17.9	44.1 ± 6.6

Infection rate is presented as the mean and standard error for 2 experiments.
OKw-312 : *1 Log, after 5days in culture ; *2 Stationary, after 15days in culture
OK-312 : *3 Log, after 10days in culture ; *4 Stationary, after 30days in culture

Algae-free paramecia are known to re-establish symbiosis when symbiotic algae are present; however, it is not clear whether they show the same reinfection rate at all periods of the growth phase. Homogenate of green paramecia including symbiotic algae was added to algae-free paramecia (Table 1). When symbiotic

algae obtained from OK-312 at log phase (Fig. 3A) were added to OKw-312 at log phase, the highest reinfection rate (66.9 ± 4.8%) was observed. In contrast, when symbiotic algae obtained from OK-312 at stationary phase (Fig. 3B) were added to OKw-312 at stationary phase, a low infection rate (44 ± 6.6%) was observed.

The relation between this morphological change and the reinfection rate requires further analysis. In addition, as the algae in *P. bursaria* may not be composed of a single species, the studies using cloned symbiotic algae are necessary to elucidate the reinfection mechanism. We are now cloning and characterizing symbiotic algae from *P. bursaria*.

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