Original

A bacteria-free monoxenic culture of *Paramecium bursaria*: its growth characteristics and the re-establishment of symbiosis with *Chlorella* in bacteria-free conditions

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SUMMARY

A bacteria-free monoxenic culture of *Paramecium bursaria* has been established. *Chlorogonium elongatum* was used as the sole food source and large numbers of easily maintained *P. bursaria* were obtained. Additional enhancement of the growth of *P. bursaria* was achieved by supplementing the culture medium with soybean lecithin. The ability of monoxenically cultured *P. bursaria* to re-establish its symbiotic relationship with *Chlorella* in bacteria-free conditions was examined. Bacteria-free, monoxenic, *Chlorella*-free (white) *P. bursaria,* and axenic isolated symbiotic *Chlorella* cultures were established from bacteria-free monoxenic cultures of *Chlorella*-bearing (green) *P. bursaria*. When the white paramecia and isolated *Chlorella* were mixed in bacteria-free conditions, they re-established a symbiotic relationship, and normal green paramecia were reconstructed. This newly developed bacteria-free monoxenic culturing of *P. bursaria* will be useful in the study of protozoan symbiosis, as this method provides us with large numbers of cells that possess the ability to re-establish a symbiotic relationship with *Chlorella*.

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INTRODUCTION

The ciliate *Paramecium bursaria* harbors many symbiotic cells of the unicellular alga *Chlorella* (Chlorophyta) in its cytoplasm. *P. bur-*

saria can be freed of its symbiotic *Chlorella* by cultivation in prolonged darkness with an excess food supply (Siegel, 1960), or by treating cells with photosynthesis inhibitors such as DCMU and methylviologen (Reisser, 1976; Hosoya et al., 1995) or with the antibiotic cycloheximide (Weis, 1984). Symbiotic *Chlorella* can also be isolated from host *P. bursaria* (Loefer, 1936; Siegel, 1960; Weis, 1978; Takeda, 1995; Nishihara et al., 1998). Thus, under laboratory conditions, both *Chlorella*-free (white) *P. bursaria* and isolated symbiotic *Chlorella* can be cultured independently. They can re-establish the symbiotic relationship when mixed, resulting in reconstruction of green paramecia (Siegel, 1960; Karakashian, 1963; Bomford, 1965; Karakashian, 1975; Weis, 1978; Weis and Ayala, 1979; Görtz, 1982; Reisser et al., 1982; Meier and Weissner, 1988; Nishihara et al., 1996; Takeda et al., 1998). Additionally, the white paramecium can be artificially infected with other microorganisms such as *Scenedesmus* (a unicellular alga), yeasts, and bacteria (Bomford, 1965; Görtz, 1982). The mechanism of the infection by microorganisms and subsequent establishment of a symbiotic relationship is still not understood. In particular, investigations of the molecular basis of symbiosis have not been possible because of difficulties in preparing the large numbers of *P. bursaria* cells essential for biochemical and molecular biological experiments.

Axenic cultures of *P. bursaria* have been described in the literature, although they only grow slowly (Loefer, 1936), or need time-consuming and rather elaborate preparations such as using glutaraldehyde-killed bacteria in the culture medium (Weis, 1975). *P. bursaria* has therefore been mainly cultured in non-sterile hay or lettuce infusions that are inoculated with food bacteria in advance (Sonneborn, 1950). Although cultures including living bacteria as a food source can provide a good cell harvest, such cultures can be easily contaminated. Especially in cultures of white *P. bursaria*, cells are more vulnerable to accidental infection by undesirable microorganisms (Görtz, 1982). Therefore, a relatively easily maintained, bacteria-free culture has long been needed for the cultivation of *P. bursaria*.

In this study, we succeeded in establishing a bacteria-free monoxenic culture with the flagellated unicellular alga, *Chlorogonium elongatum* (Chlorophyta), as a sole food source. We have characterized some features of this novel culture method, and further examined the ability of *P. bursaria* to re-establish its symbiotic relationship with *Chlorella* in bacteria-free conditions.

MATERIALS AND METHODS

Conventional culture of *P. bursaria*

Paramecium bursaria (neither syngen nor mating type identified) collected from a small pond on the campus of Kobe University (Kobe, Japan) was pre-cultured in 0.01% Knop medium (0.24 mM $Ca(NO_3)_2$, 0.14 mM KNO_3, 0.06 mM $MgSO_4$, 0.10 mM KH_2PO_4) with 10 mM HEPES-KOH (pH 7.0) at 23°C under a 12:12 h light : dark cycle, and *C. elongatum* was added as a food source. *C. elongatum* was routinely cultured in an axenic medium (*Chlorogonium* medium (CM): 1.36 mM $CaCl_2$, 0.24 M sodium acetate, 0.02% polypeptone, 0.04% tryptone and 0.04% yeast extract) at 20°C under continuous light (Sakaguchi and Suzaki, 1999).

Establishment of a bacteria-free monoxenic culture of P. bursaria

Paramecium bursaria that had been pre-cultured as described above was washed by transferring cells with a fine-tipped Pasteur pipette through about 15 ml of sterilized 0.01% Knop with 10 mM HEPES-KOH (pH 7.0) (KH) in successive Petri dishes. About 20 cells were chosen from the pre-culture, washed, and finally transferred to a dish that contained sterilized KH with antibiotics that was then sealed with Parafilm. The optimal

concentration was determined for the antibiotics penicillin, kanamycin and streptomycin. *P. bursaria* was able to increase in number, consuming *C. elongatum,* in KH that contained penicillin at concentrations less than 800 μg/ml. Kanamycin or streptomycin, even at concentrations less than 50 μg/ml, inhibited the proliferation of *P. bursaria* significantly. Therefore, 800 μg/ml penicillin was used in the experiments. The dish with penicillin and washed *P. bursaria* was incubated for several days to allow *P. bursaria* to increase in number sufficiently for the next washing procedure. Cells were washed again in sterilized KH and incubated with penicillin as described. After each incubation with penicillin, the dish was observed with an inverted light microscope, and the procedure of washing and incubation was repeated until no contaminating bacteria were observed in the dish. A part of *P. bursaria* was thereby sterilized, and was finally transferred to a penicillin-free medium consisting of 0.01% Knop and 20% CM. An adequate amount of axenically grown *C. elongatum* was added as a food source for the *P. bursaria*. The culture was incubated at 23°C under a 12:12 h light : dark cycle (light intensity, 2,000-3,000 lux), and subcultured weekly. The rest of *P. bursaria* was again subjected to the washing procedure, and a part of the culture was successively transferred to another penicillin-free culture medium. Cultures were routinely checked for sterility by spreading a drop of the culture medium onto an agar plate (2% agar in culture medium) and checking for contamination after the plate was incubated. When any contamination was found, the culture was discarded. To enhance the final harvest of *P. bursaria,* the medium composition was modified by increasing the concentration of CM or adding soybean lecithin (see details in Table 1). The axenic culture medium for *P. multimicronucleatum* used by Fok and Allen (1979) was also tested as an alternative medium, without added *C. elongatum*. To do this, *P. bursaria* from an aged culture, in which all food *C. elongatum* was consumed, was inoculated directly to the axenic medium, and then incubated at 25°C under 12:12 h light : dark cycle.

Establishment of a bacteria-free culture of white *P. bursaria* and an axenic culture of isolated symbiotic *Chlorella*

A photosynthesis inhibitor, methylviologen (1, 1-dimethyl-4, 4-bipyridinium dichloride, Sigma) was aseptically added through a syringe fitted with a disposable sterilized filter (pore size: 0.45 μm) to culture medium (0.01% Knop + 20% CM) in which green *P. bursaria* had been incubated for 7 days. The concentration of methylviologen that is enough to remove all the symbiotic *Chlorella* was preliminarily determined to be 10 μg/ml. At this concentration, no symbiotic *Chlorella* cells were observed in the host *P. bursaria* after the paramecium was exposed to the reagent for 10 days. This *Chlorella*-free (white) *P. bursaria* was subcultured in the same culture medium as that used for green *P. bursaria.* To isolate symbiotic *Chlorella*, green *P. bursaria* in an aged culture where all food *C. elongatum* had been consumed was sonicated aseptically in a small sterilized test tube, and transferred directly to an axenic culture medium for green algae (CA medium; Nishihara et al., 1998). Symbiotic *Chlorella* thereby isolated was subcultured weekly in CA medium and grown at 25°C under continuous light.

Assessment of the ability of white *P. bursaria* to re-establish the symbiotic relationship with *Chlorella* in bacteria-free culture

White *P. bursaria* was taken from a monoxenic culture in which all food *C. elongatum* had been digested. This corresponds to the 7th or 8th day of culture as shown in Fig. 2. At this stage, the white *P. bursaria* was starved but still actively swimming. Isolated symbiotic *Chlorella* were prepared from an axenic culture in its early stationary phase. White *P. bursaria* was centrifugally

(200 *g*, 5 min) washed twice in sterilized KH, as was symbiotic *Chlorella* (3,000 *g*, 5 min ×2). They were then mixed well in a tube and incubated at 20° C under continuous light. The final density of *Chlorella* cells in the mixture was 10^6 cells/ml, and the ratio of *Chlorella* to *Paramecium* was 10,000:1. After 24 hr, the mixture was washed with sterilized KH by passing the cells through a nylon mesh (11 μm pore size) to remove free *Chlorella* cells. *P. bursaria* cells retained on the mesh were recovered and transferred to 0.01% Knop + 20% CM with added *C. elongatum*. The culture was then incubated at 23°C under 12:12 h light : dark cycle. All procedures (including centrifugation, washing and transfer of cells) were carried out aseptically.

Cultures were routinely checked for sterility by spreading a drop of the culture medium onto an agar plate (2% agar and culture medium), and checking for contamination after the plate was incubated under an appropriate condition.

RESULTS

Green *P. bursaria* was readily sterilized by washing and penicillin treatment. Sterilized *P. bursaria* was inoculated, with food *C. elongatum,* into culture medium consisting of 0.01% Knop + 20% CM, and subsequently subcultured in the same medium. In this culture medium, *P. bursaria* was able to increase in number by consuming *C. elongatum*, and reached peak density after about one week. *P. bursaria* grown in this bacteria-free monoxenic culture retained several hundred healthy -looking *Chlorella* in the cytoplasm of each cell (Fig. 1). No contaminating bacteria or fungi were observed either in the culture solution or on solidified medium enriched with organic components to facilitate their growth.

In the bacteria-free monoxenic culture, peak cell density was observed first for *C. elongatum*, followed by free-living *Chlorella* cells, and then *P. bursaria* (Fig. 2). The populations of *C. elongatum* and free-living *Chlorella* were only transient; they disappeared as the growth of *P. bursaria* proceeded. The population of *P. bursaria* remained at a plateau level for at least a few weeks even without any additional food supply, and the population of free-living *Chlorella* gradually increased again. The retention of the plateau level of *P. bursaria* was dependent on the illumination conditions (Fig. 3),

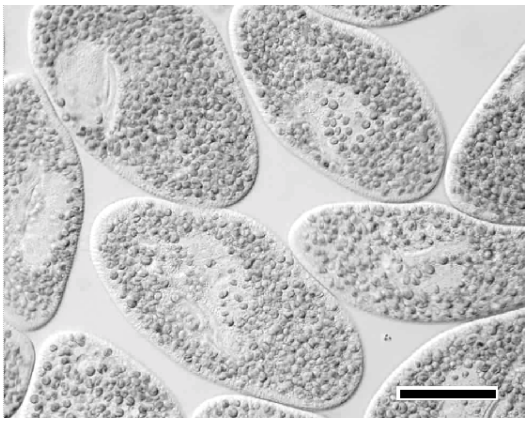

Fig. 1. *Paramecium bursaria* grown for 1 week in a bacteria-free monoxenic medium consisting of 0.01% Knop with 20% CM. Scale bar = 50 μm.

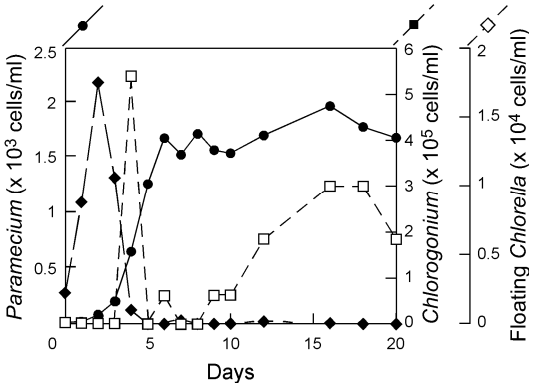

Fig. 2. A growth curve of *P. bursaria* in a bacteria-free monoxenic medium consisting of 0.01% Knop with 20% CM. The profile and the time shift of the growth curve of each population are similar at each subculture.

Table 1. Cell density (cells/ml) of *P. bursaria* in the media of different compositions.

Medium composition	0.01% Knop with			
	20% CM (a)	30% CM (b)	40% CM (c)	20% CM + 0.125 mg/ml soybean lecithin (d)
Cell density	1,500	2,000	2,500	5,000
Accumulation of *Chlorella*	+	++	+++	+

Final cell densities were determined at 7 days post-inoculation for a–c and 11 days for d. The relative amount of accumulated free-living *Chlorella* in the medium is indicated by numbers of "+" marks (+, ~10^4 cells/ml; ++, ~10^5 cells/ml; +++, ~10^6 cells/ml).

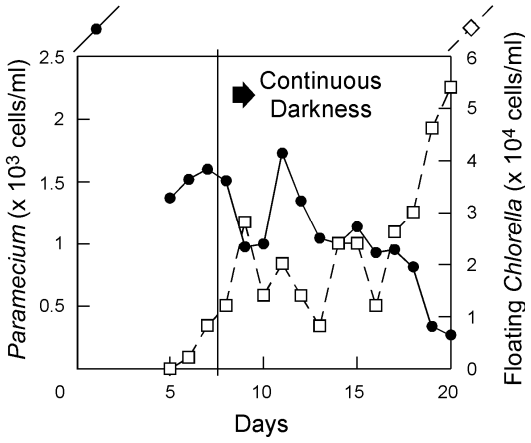

Fig. 3. A population profile of green *P. bursaria* in the dark. The medium consists of 0.01% Knop with 20% CM. From day 8 after inoculation, the culture flask was incubated in the dark.

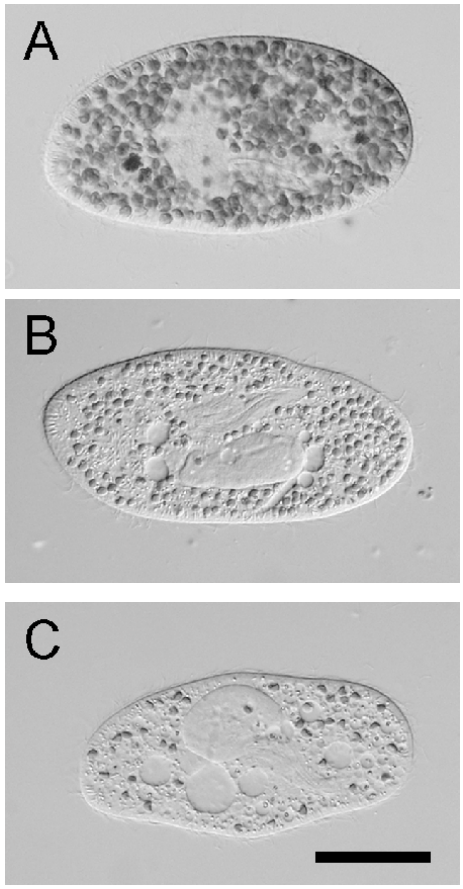

Fig. 4. Light micrographs of green *P. bursaria* taken from the culture shown in Fig. 3. A: day 7. B: day 14. C: day 20. Scale bar = 40 μm.

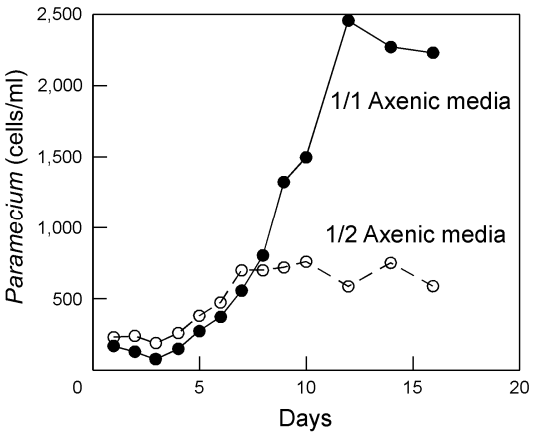

Fig. 5. Growth curves of *P. bursaria* in the complete axenic medium for *P. multimicronucleatum* at full and half strength. In the full-strength medium (closed circles), the cell density reached a maximum of 2,500 cells/ml at 12 days after the inoculation, while smaller numbers of cells were harvested in half-strength medium (open circles).

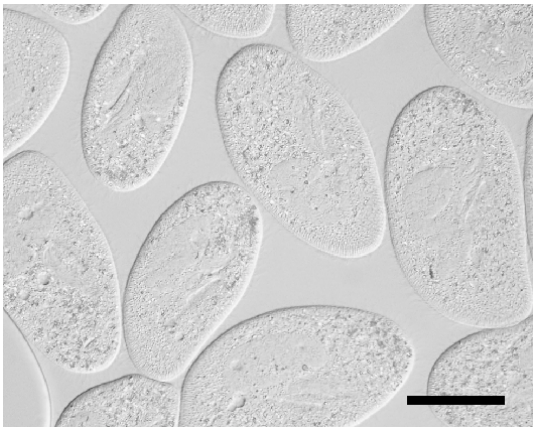

Fig. 6. White *P. bursaria* grown for 1 week in a bacteria-free monoxenic culture medium consisting of 0.01% Knop with 20% CM. Food *C. elongatum* had been completely digested and the cells were slightly starved. Scale bar = 50 μm.

indicating that photosynthesis of symbiotic *Chlorella* contributes to survival of the host cell in this bacteria-free culture medium. Microscope observations on *P. bursaria* kept in the dark showed that the host paramecium gradually lost its symbiotic *Chlorella* (Fig. 4, A-C), suggesting that the symbiotic *Chlorella* was digested as an innate food source.

The peak cell density of green *P. bursaria* was increased by raising the concentration of CM in the culture medium (Table 1). The higher the concentration of CM, the more the growth of *C. elongatum*, providing more food for *P. bursaria* and resulting in higher peak cell densities at 7–8 days after inoculation. At concentrations of CM above 30%, increased death of *P. bursaria* was observed during its growth phase (data not shown), along with an accumulation of free-living *Chlorella* cells in the medium after the growth of *P. bursaria* reached its plateau level. In this situation, the culture medium appeared green due to the accumulated *Chlorella* cells, and the survival period of *P. bursaria* was much shorter (data not shown). The optimum concentration of CM is therefore 20%.

Addition of soybean lecithin to the culture medium facilitated the growth of *P. bursaria* (Table 1). Although a few additional culture days were required to reach the peak cell density, *P. bursaria* remained at the plateau level for a few weeks as it did in the lecithin-free medium. In the presence of lecithin, however, accumulation of free-living *Chlorella* cells was not observed during the plateau phase. The opaque appearance of the culture medium due to the added lecithin gradually faded through the incubation period, and the medium cleared completely 1–2 days after all food *C. elongatum* were taken up by *P. bursaria*.

Further improvement of cell harvest was expected in axenic culture conditions already developed for other species of the genus *Paramecium* including *P. multimicronucleatum*. *P. bursaria* increased in number in the axenic medium developed for *P. multimicronucleatum*, and the cell density of *P. bursaria* in this medium finally reached

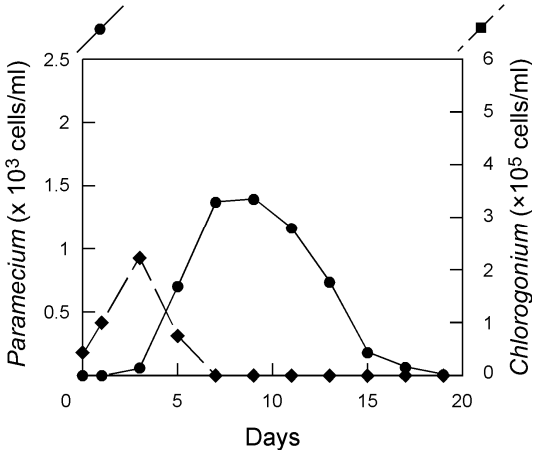

Fig. 7. A growth curve of white *P. bursaria* in a bacteria-free monoxenic medium in 0.01% Knop with 20% CM. In contrast to the *Chlorella*-bearing normal *P. bursa*ria shown in Fig. 2, the cell density of *P. bursaria* gradually decreased after it reached its peak at 7–10 days after inoculation. The profile and the time shift of the growth curve of each population are similar at each subculture.

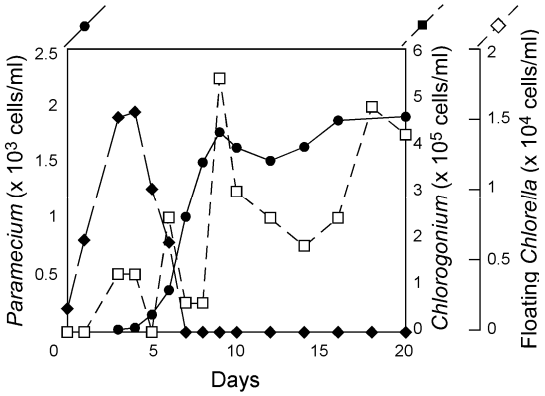

Fig. 9. Growth of white *P. bursaria* that was incubated with *Chlorella* for 24 h. The medium consists of 0.01% Knop solution with 20% CM.

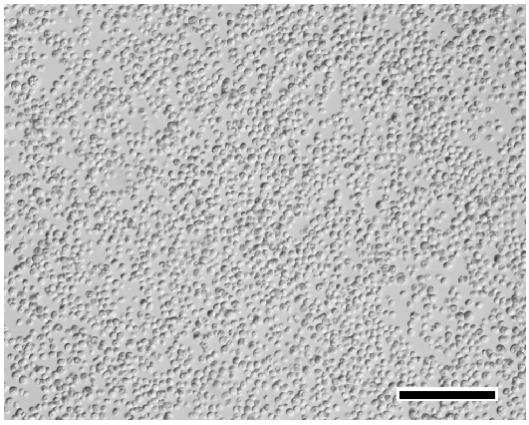

Fig. 8. Isolated symbiotic *Chlorella* grown in CA medium. Scale bar = 50 μm.

about 2,500 cells/ml 12 days after inoculation (Fig. 5).

Chlorella-free (white) *P. bursaria* was prepared aseptically by exposing green *P. bursaria* cultured in sterile monoxenic medium to the photosynthesis inhibitor methylviologen. During the exposure period, the paramecium gradually lost its symbiotic *Chlorella* cells and, 10 days after the treatment, no symbiotic *Chlorella* at all was observed in the host cells. *P. bursaria* thereby freed from symbiotic *Chlorella* was cultured with food *C. elongatum* in the bacteria-free monoxenic medium used for green *P. bursaria*. In this culture, no *Chlorella* was observed either in the host cell or in the surrounding medium through successive subcultures, indicating that the paramecia were completely deprived of their symbiotic *Chlorella* (Fig. 6). The population density of white *P. bursaria* increased and reached its peak cell density 6–7 days after inoculation, and gradually decreased following a stationary period of about 2 days (Fig. 7). The resulting peak cell density was almost identical to that of green *P. bursaria* (Fig. 2). An axenic culture of isolated symbiotic *Chlorella* was also successfully established directly from sonicated green *P. bursaria* (Fig. 8). The population of isolated symbiotic *Chlorella* reached about 7×10^6 cells/ml within 2 weeks under continuous light.

The ability of white *P. bursaria* to re-establish symbiosis with *Chlorella* was assayed in bacteria-free conditions. White *P. bursaria* that was fed on *Chlorella* cells for 24 hr also increased in number in the bacteria-free monoxenic medium

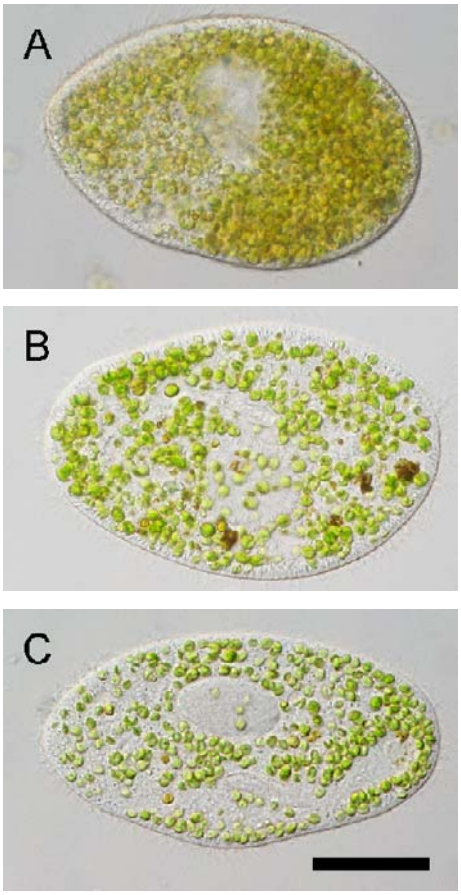

Fig. 10. Light micrographs of *P. bursaria* taken from the culture shown in Fig. 9. A: day 0. B: day 8. C: day 14. Scale bar = 40 μm.

(Fig. 9). The population of *Chlorella*-fed white *P. bursaria* increased and reached peak cell density in a similar manner to both green and white *P. bursaria*, and afterwards did not decrease, but retained a plateau level. Light microscope observations on *Chlorella*-fed white *P. bursaria* were made during the culture period. On day 0, just after the 24-hour incubation with *Chlorella* finished, the cytoplasm of the paramecia was filled with abundant ingested *Chlorella* cells. Many of the *Chlorella* cells were being digested, and the paramecia appeared brownish (Fig. 10A). The paramecia indeed ejected mixtures of digested (brown) and apparently healthy (green) *Chlorella* cells during observation. On day 8 after the inoculation, all food *C. elongatum* were consumed, and the cytoplasm of the paramecia had cleared, leaving many healthy-looking green *Chlorella* cells stabilized in the peripheral region of the host paramecia (Fig. 10B). On day 14, after the population of *Chlorella*-fed white *P. bursaria* reached a plateau level, many green *Chlorella* cells were still observed in the paramecia (Fig. 10C). This *Chlorella*-fed white *P. bursaria* was successively subcultured in the same medium. The growth curve of established *Chlorella*-fed white *P. bursaria* was identical to that of green *P. bursaria*, and no white paramecia emerged in the culture medium even after prolonged subculturing (data not shown).

DISCUSSION

In this study, bacteria-free monoxenic cultures of *P. bursaria* was established, using *C. elongatum* as a food source. Peak cell density was reached first by *C. elongatum*, followed by *Chlorella* growing free in the medium, and then *P. bursaria* reached its peak as the other two populations declined. The profile of the growth curve was identical for green and white *P. bursaria*. This indicates that, at least in the presence of sufficient external food, the growth of *P. bursaria* is not influenced by factors derived from its symbiotic *Chlorella*. Similar growth profiles for *P. bursaria* have been reported earlier (Karakashian, 1963; Pado, 1965; Karakashian, 1975; Nishihara et al, 1996), although they were not grown in bacteria-free conditions. After all food *C. elongatum* was consumed, white *P. bursaria* decreased in number, but green *P. bursaria* retained its cell density at a plateau level for a few weeks. In the dark, however, *Chlorella*-bearing *P. bursaria* decreased in number, with a gradual loss of symbiotic intracellular

Chlorella. Symbiotic *Chlorella* releases the major part of newly-fixed photosynthetic products as maltose (Muscatine et al., 1967; Ziesenisz et al., 1981; Dorling et al., 1997), and the host paramecium appears to use it (Muscatine et al., 1967; Brown and Nielsen, 1974). After all external food was consumed in the bacteria-free culture, the survival of *P. bursaria* was dependent on the presence of intracellular symbiotic *Chlorella*, which probably provides photosynthetic products in the light, and may be digested by the host paramecium in the dark.

For the first time, the population of *Chlorella* living free in the surrounding medium was investigated throughout the culture cycle. Free-living *Chlorella* floating in the medium has been reported in an aged culture of *P. bursaria* in non-sterile conditions (Siegel, 1960). It is speculated that the free-floating *Chlorella* may have been liberated from dead host cells or released from intact host cells, although the origin of the algae was not identified. We observed green *P. bursaria* exocytotically releasing living *Chlorella* cells from the cytoproct after feeding on food *C. elongatum* (not shown). This phenomenon is under further study and will be reported elsewhere. In our bacteria-free culture, the cell density of free-living *Chlorella* first increased and then decreased markedly during the early growth phase of *P. bursaria*, strongly suggesting that *P. bursaria* took up the free *Chlorella* cells. Weis (1976) showed that *Chlorella*-bearing *P. bursaria* can grow by feeding on extracellular *Chlorella* from the surrounding medium, and about 2,000 *Chlorella* cells per paramecium per day are required to allow growth with a generation time of 40 hr. In our bacteria-free culture, the ratio of free-living *Chlorella* cells to green *P. bursaria* cells was, at most, 10 at the transient peak cell density of free-living *Chlorella* (for example, at day 4 in Fig. 2). Therefore, in our cultures, free-living *Chlorella* would not contribute substantially to the growth of *P. bursaria,* even if they were all digested.

The composition of our culture medium was modified to improve the final cell harvest of *P. bursaria*. At higher concentrations of CM, the peak cell density of *P. bursaria* increased, although excess free-living *Chlorella* accumulated. This accumulation could be due to increased exocytotic release of intracellular *Chlorella* cells from living paramecia, but is more likely due to release from dead *P. bursaria* cells, which were found frequently on the bottom of the culture vessel. Concentrated CM may have direct detrimental effects on *P. bursaria*, or may contribute to excess growth of existing free *Chlorella,* which might then excrete factors fatal to *P. bursaria*. We did not investigate this further because we found that addition of soybean lecithin was far superior for increasing paramecium growth. When soybean lecithin was added to the medium, *P. bursaria* underwent additional fissions, and maintained its cell density at a plateau level without excess accumulation of free-living *Chlorella*. Lecithin, phosphatidylethanolamine, or phosphatidylcholine has been previously used as a lipid source in axenic media for *Paramecium* (Fok and Allen, 1979; Schönefeld et al., 1986). Weis (1975) grew *P. bursaria* using an exogenous lipid source in an axenic medium. When our culture medium was supplemented with soybean lecithin, *P. bursaria* was probably able to use this as a source of lipid to achieve additional growth.

We also confirmed that *P. bursaria* is able to grow without food *C. elongatum* in a completely axenic medium that was originally developed for *P. multimicronucleatum* (Fok and Allen, 1979). However, the final cell density, 2,500 cells/ml, was only the same as that obtained in our medium without soybean lecithin. Considering the elaborate preparation procedures and complicated constituents for this complete axenic medium, we believe that our monoxenic culture method, with food *C. elongatum,* is superior to the axenic method for culturing large quantities of *P. bursaria*. Different strains and species of *Paramecium* have different

nutritional requirements, and the amounts of, as well as the ratios between, phospholipids and stigmasterol have been regarded as critical for ensuring optimum growth of *Paramecium* (Soldo and van Wagtendonk, 1969; Fok and Allen, 1979; Hennessey et al., 1983). Weis (1975) showed that, in the axenic medium, *P. bursaria* requires a certain ratio between TEM-4T (replaceable with phospholipids) and stigmasterol for optimal growth. Therefore, further modifications to both the axenic and our monoxenic medium would possibly promote better growth of *P. bursaria*.

Using a bacteria-free monoxenic culture of white *P. bursaria* and an axenic culture of isolated symbiotic *Chlorella*, the ability of white *P. bursaria* to re-establish a symbiotic relationship with its original symbiotic *Chlorella* was assayed in bacteria-free conditions. We employed two criteria to judge the success of re-establishment: the morphology and the growth curve of *Chlorella*-fed white *P. bursaria*. In normal *P. bursaria*, symbiotic *Chlorella* cells are stabilized in the peripheral region of the host cell (Karakashian et al. 1968; Weis 1976), and *Chlorella* thereby appears to escape the host's digestive process (Karakashian and Rudzinska, 1981; Karakashian and Karakashian, 1973). The peripheral region of *P. bursaria* cells is, however, also able to harbor some species of bacteria and fungi that do not provide the host cell with any benefits (Görtz, 1982). Therefore, the retention of ingested *Chlorella* in the peripheral region of the host cell seems not to be the result of re-established symbiosis, but is a prerequisite for it. Confirmation of the symbiotic relationship should be possible by observing characteristics that occur only in *Chlorella*-bearing *P. bursaria*. In our bacteria-free monoxenic culture medium, *Chlorella*-fed white *P. bursaria* accumulated many *Chlorella* cells in the peripheral region, and maintained its cell density at a plateau level as green *P. bursaria* does. This implies that photosynthetic products from established intracellular symbiotic *Chlorella* are being used by the paramecium, indicating that a symbiotic relationship was successfully re-established between white *P. bursaria* and *Chlorella*. This result also demonstrates that the symbiotic relationship between *P. bursaria* and its *Chlorella* does not involve any other organisms, such as co-existing bacteria.

We conclude that our bacteria-free monoxenic culture method for *P. bursaria* can provide enough cells that are able to re-establish and maintain a symbiotic relationship with *Chlorella* to enable further studies on the molecular mechanism of the symbiotic relationship between *P. bursaria* and *Chlorella.*

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REFERENCES

Bomford, R. (1965) Infection of alga-free *Paramecium bursaria* with strains of *Chlorella*, *Scenedesmus*, and a yeast. J. Protozool., 12, 221-224.

Brown, J. A. and Nielsen, P. J. (1974) Transfer of photosynthetically produced carbohydrate from endosymbiotic *Chlorella* to *Paramecium bursaria*. J. Protozool., 21, 596-570.

Dorling, M., McAuley, P. J. and Hodge, H. (1997) Effect of pH on growth and carbon metabolism of maltose-releasing *Chlorella* (Chlorophyta). Eur. J. Physiol., 32, 19-24.

Fok, A. K. and Allen, R. D. (1979) Axenic *Para-*

mecium caudatum. I. Mass culture and structure. J. Protozool., 26, 463-470.

Görtz, H.-D. (1982) Infection of *Paramecium bursaria* with bacteria and yeasts. J. Cell Sci., 58, 445-453.

Hennessey, T. M., Andrews, D. and Nelson, D. L. (1983) Biochemical studies of the excitable membrane of *Paramecium tetraurelia*. VII. Sterols and other neutral lipids of cell and cilia. J. Lipid Res., 24, 575-587.

Hosoya, H., Kimura, K., Matsuda, S., Kitaura, M., Takahashi, T. and Kosaka, T. (1995) Symbiotic algae-free strains of the green paramecium *Paramecium bursaria* produced by herbicide paraquat. Zool. Sci., 12, 807-810.

Karakashian, S. J. (1963) Growth of *Paramecium bursaria* as influenced by the presence of algal symbionts. Physiol. Zool., 36, 52-68.

Karakashian, M. W. (1975) Symbiosis in *Paramecium bursaria*. Symp. Soc. Exp. Biol., 29, 145-173.

Karakashian, M. W. and Karakashian, S. J. (1973) Intracellular digestion and symbiosis in *Paramecium bursaria*. Exp. Cell Res., 81, 111-119.

Karakashian, S. J., Karakashian, M. W. and Rudzinska, M. A. (1968) Electron microscopic observations on the symbiosis of *Paramecium bursaria* and its intracellular algae. J. Protozool., 15, 113-128.

Karakashian, S. J. and Rudzinska, M. A. (1981) Inhibition of lysosomal fusion with symbiont -containing vacuoles in *Paramecium bursaria*. Exp. Cell Res., 131, 387-393.

Loefer, J. B. (1936) Bacteria-free culture of *Paramecium bursaria* and concentration of the medium as a factor in growth. J. Exp. Zool., 72, 387-407.

Meier, R. and Wiessner, W. (1988) Infection of algae-free *Paramecium bursaria* with symbiotic *Chlorella* sp. isolated from green paramecia. I. Effect of the incubation period. Europ. J. Protistol., 24, 69-74.

Muscatine, L., Karakashian, S. J. and Karakashian, M. W. (1967) Soluble extracellular products of algae symbiotic with a ciliate, a sponge and a mutant hydra. Comp. Biochem. Physiol., 20, 1-12.

Nishihara, N., Takahashi, T., Kosaka, T. and Hosoya, H. (1996) Characterization of symbiotic algae-free strains of *Paramecium bursaria* produced by the herbicide paraquat. J. Protozool. Res., 6, 60-67.

Nishihara, N., Horiike, S., Takahashi, T., Kosaka, T., Shigenaka, Y. and Hosoya, H. (1998) Cloning and characterization of endosymbiotic algae isolated from *Paramecium bursaria*. Protoplasma, 203, 91-99.

Pado, R. (1965) Mutual relation of Protozoans and symbiotic algae in *Paramecium bursaria*. I. The influence of light on the growth of symbionts. Folia Biol., 13, 173-182.

Reisser, W. (1976) Die stoffwechselphysiologischen Beziehungen zwischen *Paramecium bursaria* Ehrbg. und *Chlorella* spec. in der *Paramecium bursaria*-Symbiose. I. Der Stickstoff- und der Kohlenstoff-Stoffwechse. Arch. Microbiol., 107, 357-360.

Reisser, W., Radunz, A. and Wiessner, W. (1982) Participation of algal surface structure in the cell recognition process during infection of aposymbiotic *Paramecium bursaria* with symbiotic chlorellae. Cytobios, 33, 39-50.

Sakaguchi, M. and Suzaki, T. (1999) Monoxenic culture of the heliozoon *Actinophrys sol*. Europ. J. Protistol., 35, 411-415.

Schönefeld, U., Alfermann, A.W. and Schultz, J. E. (1986) Economic mass cultivation of *Paramecium tetraurelia* on a 200-liter scale. J. Protozool., 33, 222-225.

Siegel, R. W. (1960) Hereditary endosymbiosis in *Paramecium bursaria*. Exp. Cell Res., 19, 239-252.

Soldo, A. T. and Van Wagtendonk, W. J. (1969)

The nutrition of *Paramecium aurelia*, stock 299. J. Protozool., 16, 500-506.

Sonneborn, T. M. (1950) Methods in general biology and genetics of *Paramecium Aurelia*. J. Exp. Zool., 113, 87-148.

Takeda, H. (1995) Cell wall composition and taxonomy of symbiotic *Chlorella* from *Paramecium* and *Acanthocystis*. Phytochem., 40, 457-459.

Takeda, H., Sekiguchi, T., Nunooka, S. and Usuki, I. (1998) Species-specificity of *Chlorella* for establishment of symbiotic association with *Paramecium bursaria* - Does infectivity depend upon sugar components of the cell wall? Europ. J. Protozool., 34, 133-137.

Weis, D. S. (1975) A medium for the axenic culture of *Chlorella*-bearing *Paramecium bursaria* in the light. Trans. Amer. Micro. Soc., 94, 109-117.

Weis, D. S. (1976) Digestion of added homologous algae by *Chlorella*-bearing *Paramecium bursaria*. J. Protozool., 23, 527-529.

Weis, D. S. (1978) Correlation of infectivity and concanavalin A agglutinability of algae exsymbiotic from *Paramecium bursaria*. J. Protozool., 25, 366-370.

Weis, D. S. (1984) The effect of accumulation time of separate cultivation on the frequency of infection of aposymbiotic ciliates by symbiotic algae in *Paramecium bursaria*. J. Protozool., 31, 13A-14A.

Weis, D. S. and Ayala, A. (1979) Effect of exposure period and algal concentration on the frequency of infection of aposymbiotic ciliates by symbiotic algae from *Paramecium bursaria*. J. Protozool., 26, 245-248.

Ziesenisz, E., Reisser, W. and Weissner, W. (1981) Evidence of de novo synthesis of maltose excreted by the endosymbiotic *Chlorella* from *Paramecium bursaria*. Planta, 153, 481-485.