Symbiosis, 34 (2003) 203–214
Balaban, Philadelphia/Rehovot

Culture and Pyrenoid Structure of a Symbiotic *Chlorella* Species Isolated from *Paramecium bursaria*

MIHO NAKAHARA[1*], SHINJI HANDA[2], TAKETO NAKANO[3], and HIRONORI DEGUCHI[1]

[1]*Department of Biological Science, Graduate School of Science, Hiroshima University, Kagamiyama 1-3-1, Higashi-Hiroshima-shi, Hiroshima-ken 739-8526, Japan, Tel. +81-824-247452, Fax. +81-824-247252, Email. ousama@hiroshima-u.ac.jp;*
[2]*Hiroshima Environment and Health Association, Hirosekita-machi 9-1, Naka-ku, Hiroshima-shi, Hiroshima-ken 730-8631, Japan, Tel. +81-82-2931517, Fax. +81-82-2935049, Email. shinji.handa@kanhokyo.or.jp; and*
[3]*Hiroshima Institute of Technology, Saeki-ku, Hiroshima-shi, Hiroshima-ken 731-5193, Japan, Tel. +81-82-9213121, Fax. +81-82-9234681*

Received December 21, 2002; Accepted May 24, 2003

Abstract

The pyrenoid structure has been regarded as a stable feature worthy of taxonomic importance in defining species or genera for unicellular free-living or symbiotic green algae of the ciliate, *Paramecium bursaria*. We compared the pyrenoids of a *Chlorella* symbiotic association in *P. bursaria* with those in cells cultured in artificial conditions after isolation from the ciliate. We did not find any difference in the morphology of the chloroplast between symbiotic and isolated cells under light microscopic (LM) observation. The axenically isolated strain was cultured on the CA medium solidified by 1.5% agar, containing NH_4NO_3 as the nitrogen source and no carbon source. Under light microscopic observation, all algae had their pyrenoids surrounded by two starch sheaths in a cup-shaped chloroplast when growing in the symbiotic condition, while every strain had no or degenerated pyrenoids without starch sheaths when grown in medium. We were

*The author to whom correspondence should be sent.

0334-5114/2003/$05.50

unable to keep the cultures of the isolates growing for longer than several months in spite of frequent transfers to fresh media. These results suggest that the symbiotic algal strains show a strong dependency on the host. Further comparative study is needed using transmission electron microscopy (TEM) for confirmation of the observations on the pyrenoid morphology made under LM.

Keywords: Symbiotic algae, *Chlorella*, pyrenoid, morphology, physiology, *Paramecium bursaria*

1. Introduction

Paramecium bursaria Ehrenberg, a freshwater ciliate, contains several hundred intracellular symbiotic coccoid green algae. The symbiotic algae are isolable from their host and can be grown in culture. Many workers have reported that the green algae isolated from the ciliates are species of *Chlorella* Beijerinck or *Chlorella*-like algae (e.g., Beijerinck, 1890; Loefer, 1936; Reisser, 1975, 1976, 1988; Siegel, 1960; Siegel and Karakashian, 1959). Reisser (1984, 1988, 1992), when discussing these symbiotic algae, regarded them as species of *Chlorella*. The true identity of the *Paramecium* alga is, however, still unclear; whether it is closely related with *C. vulgaris* ("true" *Chlorella*) or if it belongs to another genus. Furthermore, Friedl (1995) and Huss et al. (1999) showed that *Chlorella* is a polyphyletic assemblage of coccal green algae.

The pyrenoid structure has been regarded as one of the stable features useful in taxonomic recognition of species or genera for unicellular free-living or symbiotic green algae. The symbiotic algae have been described as having their single pyrenoid surrounded by two starch sheaths, with the thylakoid lamellae extending into the pyrenoid matrix. Although morphological features of these symbiotic algae resemble the free-living *Chlorella* species, their identification has not yet been supported by culture in isolated conditions or by demonstration of their life cycle.

The aim of the present study is to compare the structures of the pyrenoid of the coccoid algae in a symbiotic association with *P. bursaria*, isolated from the ciliates and cultured in artificial conditions.

2. Materials and Methods

Collection and maintenance of ciliates

The ciliate *Paramecium bursaria* was collected from a pond in Hiroshima

Prefecture, southern Japan, in 1997 and 1999. Each individual of the ciliate was isolated by the pipette washing method (Pringsheim, 1946), and cultured in a test tube containing the Sonneborn (1950) medium under the conditions of ca 20°C, 36 $\mu E/m^2/s$, 12:12 h LD cycle.

Isolation and cultivation of the symbiotic algae

The algae in symbiotic condition were observed by rupturing a single cell of the ciliate on a glass slide in one drop of sterilized water with minimal damage for the symbiotic algae. The isolated algal strains from each strain of the ciliate were obtained by the pipette washing method (Pringsheim, 1946), which is able to detect not only algae with standard growth rates but also others with low rates. Isolated algae were precultured for several weeks on agar plates in three media: BBM medium (Bischoff and Bold, 1963), C medium (Ichimura, 1971), and CA medium (Ichimura, 1974). CA medium contains NH_4NO_3 as nitrogen source compared with C medium. After initial growth on agar plates, the algal colonies were transferred to agar slants under the conditions of ca 20°C, 36 $\mu E/m^2/s$, 12:12 h LD cycle. All strains used in this study were deposited in the Department of Biological Science, Graduate School of Science, Hiroshima University, Japan.

3. Results

Isolation and cultivation of symbiotic algae

Colonies grew when cultured on C and CA 1.5% agar medium containing NH_4NO_3 as the nitrogen source and no carbon source (Table 1). The colonies on CA medium established healthy green strains, whereas the colonies on C medium did not, and the strains became pale green. No colony was observed to grow on BBM agar medium containing $NaNO_3$ as the nitrogen source and no carbon source. Isolations of the symbiotic algae were repeated six times. A total of 56 algal strains (14 and 42 strains) were obtained from the two clonal strains of *P. bursaria*: HB2-1 and HB2-2, respectively (Table 2). Only 5 to 11 strains survived at the end of the first month after isolations. However, all strains obtained in 1997–2002, except one of 11 strains obtained in 2001, disappeared within several months after isolation despite frequent transfers to fresh media. After all, three types of the symbiotic algal strains were obtained: one type could not survive the first month, another died several months after isolations, and the other survived at least 20 months (for April 1, 2003).

Table 1. Table of components.

	Medium BBM	C	CA
$NaNO_3$	+	–	–
$CaCl_2 \cdot 2H_2O$	+	–	–
$MgSO_4 \cdot 7H_2O$	+	+	+
K_2HPO_4	+	–	–
KH_2PO_4	+	–	–
NaCl	+	–	–
T-1[a]	+	–	–
T-2[b]	+	–	–
T-3[c]	+	–	–
T-4[d]	+	–	–
$Ca(NO_3)_2 \cdot 4H_2O$	–	+	+
KNO_3	–	+	+
NH_4NO_3	–	–	+
β-Na_2 glycerophosphate·$5H_2O$	–	+	+
Vitamine mix[e]	+	+	+
P-IV metals[f]	–	+	+
Fe[g]	–	–	+
Buffer	–	Tris	Hepes

a: Na_2EDTA, b: $FeSO_4 \cdot 7H_2O$, H_2SO_4; c: H_3BO_3, d: $ZnSO_4 \cdot 7H_2O$, $MnCl_2 \cdot 4H_2O$, $CuSO_4 \cdot 5H_2O$, $Co(NO_3)_2 \cdot 6H_2O$, Na_2MoO_4, H_2SO_4; e: Vitamine B12, Biotin, Thiamine HCl; f: $FeCl_3 \cdot 6H_2O$, $MnCl_2 \cdot 4H_2O$, $ZnSO_4 \cdot 7H_2O$, $CoCl_2 \cdot 6H_2O$, $Na_2MoO_4 \cdot 2H_2O$, $Na_2EDTA \cdot 2H_2O$; g: $Fe(NH_4)_2(SO_4)_2 \cdot 6H_2O$, $Na_2EDTA \cdot 2H_2O$, +: contain; –: null.

Table 2. Success of *Chlorella* isolated from different strains and collection years of *Paramecium bursaria* from Japan.

Strain of *Paramecium bursaria*	Total number of isolated algae[a]	Year isolated	Isolated algal strains of presence
HB2-1	5	1997	–
HB2-1	9	1999	–
HB2-2	9	1999	–
HB2-2	11	2000	–
HB2-2	11	2001	+ (1)
HB2-2	11	2002	+ (11)

+: Presence (a total number of cultural algal strains); –: absence for not surviving, death several months later. a) survived after one month.

Table 3. Morphological features of symbiotic, isolated and free-living algae.

Algae	Diameter of cells (μm)	Shape of cell	Number of autospores	Shape of plastid	Pyrenoid	Starch sheaths	Mother cell wall
Symbiotic condition	3.3–5.8	Spherical, ellipsoidal	2, 4	Girdle, cup	+	+	+
Isolated condition HB2-2-1	2.5–12.0	Spherical, ellipsoidal	2, 4, 8, 16	Saucer, girdle, cup	+, –	+, –	+
Free-living *Chlorella vulgaris* NIES-227*	2.5–10.0	Spherical, ellipsoidal	2, 4, 8, 16	Saucer, girdle, cup	+	+	+

*Strain code refers to NIES-Collection number of National Institute for Environmental Studies, Japan. +: presence, –: absence.

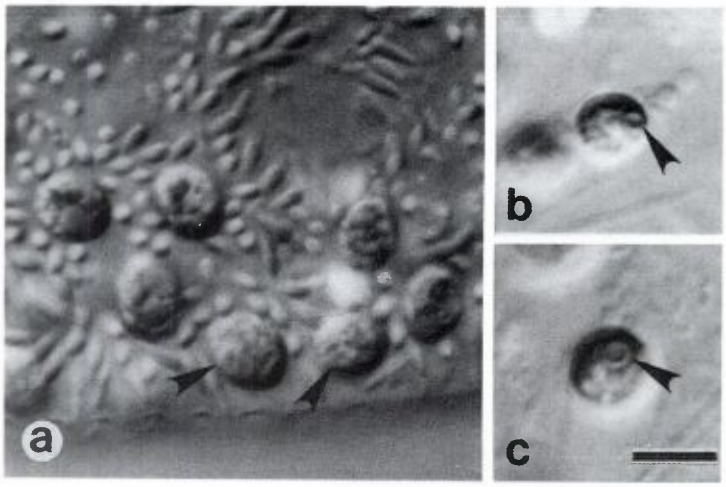

Figure 1. Symbiotic *Chlorella* algae in symbiotic condition. a) a part of *P. bursaria*, showing symbiotic *Chlorella* and organelles of a ciliate, b), c) algal cells from *P. bursaria* ruptured on a glass slide. Note that the algae have a distinct pyrenoid with obvious starch sheaths in every cell. All at the same magnification. Scale bar: 5 μm. Arrowheads indicate pyrenoids.

Figs. 2–5. Vegetative cells which die several months after isolation from *P. bursaria* in culture. Arrowheads indicate pyrenoids and arrows starch sheaths. All at the same magnification, scale bar: 5 μm.

Figure 2. Spherical cells. a) cell with distinct pyrenoids with obvious starch sheaths (4 weeks after isolation), b) as in a, but with thin starch sheaths (8 weeks after isolation), c) as in a, but pyrenoid small (10 weeks after isolation), d) as in c, but without starch sheaths, e) cell without a pyrenoid.

Figure 3. Ellipsoidal cells. a) cell with distinct pyrenoids with obvious starch sheaths (4 weeks after isolation), b) cell with small pyrenoids and thin starch sheaths (8 weeks after isolation), c) as in a, but without starch sheaths (10 weeks after isolation), d) cell without a pyrenoid. All at the same magnification.

Figure 4. Cells at two autospore formation. a) cell with a distinct pyrenoids with obvious starch sheaths (4 weeks after isolation), b) as in a, but with thin starch sheaths (8 weeks after isolation), c) as in a, but no starch sheaths, d) as in c, but small pyrenoids, e) as in d, but very small pyrenoids.

Figure 5. Cells at autospore formation. a–c) different numbers of autospores within the sporangial wall. a) four, b) eight, c) 16, d) persistant sporangial wall after release of autospores.

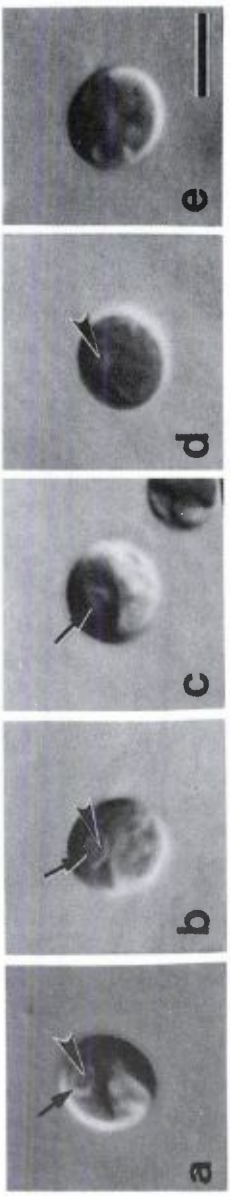

Fig. 2

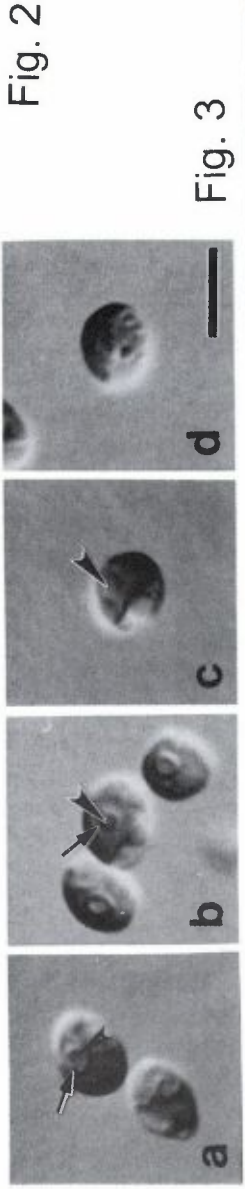

Fig. 3

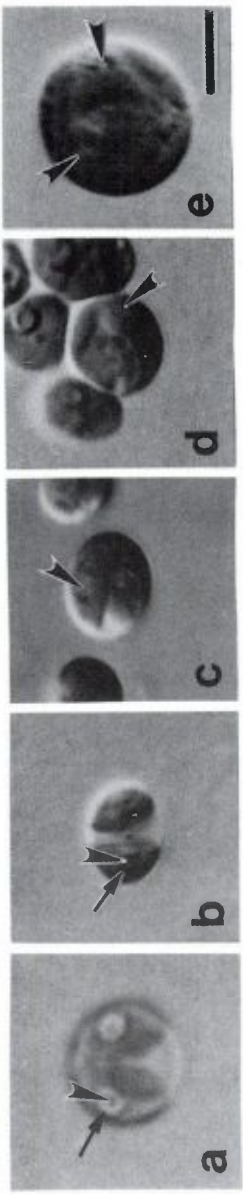

Fig. 4

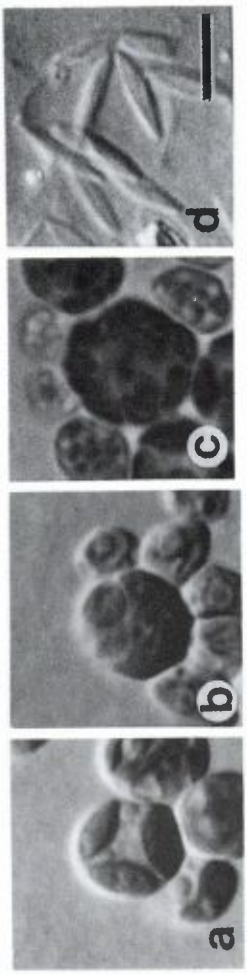

Fig. 5

Algae in symbiotic condition (Fig. 1, Table 3)

The symbiotic algae in their host from which the surviving isolated 12 strains of algae were obtained, exhibit the following features: Young cells ellipsoidal, chloroplast girdle or cup shaped; pyrenoid single, surrounded by two starch sheaths, spherical to broadly ellipsoidal (Fig. 1). Mature cells broadly ellipsoidal to spherical, chloroplast cup, girdle or saucer shaped; pyrenoid single, surrounded by two starch sheaths, spherical to broadly ellipsoidal (Fig. 1). Cell wall smooth (Fig. 1b). Reproduction was only by autospore. Cells endogenously divided into 2 or 4 autospores within the mother cell wall (Fig. 1a). Autospores were about equal in size. Zoospores were not observed in this study. Vegetative cell size was 3.3–5.8 μm in diameter.

The chloroplast has a single pyrenoid with obvious starch sheaths in every stage of the life cycle (Fig. 1). These features of the symbiotic algae are characteristics of typical *Chlorella*.

Algae growing in culture

Ten algal strains have been isolated from *Paramecium* strain HB2-2. Differences in the morphology of the pyrenoid were observed between freshly isolated cells and those cells after several months of cultivation. The following paragraph describes differences in the morphology 1–4 months after isolation (Figs. 2–5, Table 3).

Young cells ellipsoidal; chloroplast girdle or cup shaped; pyrenoid single, surrounded by two starch sheaths, spherical to broadly ellipsoidal (Figs. 2a, 2b, 3a, 4a) in cells newly isolated from *P. bursaria;* pyrenoid less distinct (Figs. 3b, 4b) and lacking starch sheaths (Figs. 2d, 3c, 4c, 4d) in most of mature cells one month later; no or degenerated pyrenoids, devoid of starch sheaths or else sheaths at different stages of degeneration (Figs. 2b–2c, 3b–3d, 4b–4e) after two months of cultivation. Cell wall smooth. Chloroplasts single and cup, saucer or girdle shaped. Vegetative cell size 2.5–7.5 μm in diameter, with old cells measuring up to 12 μm in diameter. Reproduction only by autospore formation. Cells divided into 2, 4, 8, or 16 autospores within the mother cell wall (Figs. 5a–c). Autospores about equal in size. Mother cell walls remained for a long time in culture media after releasing daughter cells (Fig. 5d). Zoospores were not observed in this study.

No differences in shape of the chloroplasts were found between the symbiotic and isolated algae (Figs. 1–4). Also, no influence on autospore formation was found in degeneration of pyrenoids (Fig. 4). This fact suggests that cells do not die by growing weaker for any apparent reason. However, the algae became pale green in color compared to those in the symbiotic condition or in newly

isolated culture. The vacuoles of the algae with reduced pyrenoids were small and few (Figs. 1–4). Finally, after several months, the algae became faded in color and no longer formed autospores.

Algal strains examined: HB2-2-1 to -11. One of the cultured, HB2-2-12, had no differences from later cells that were newly isolated.

4. Discussion

Pyrenoid structure of symbiotic Chlorella

Pyrenoid structure has been regarded as a stable feature useful in the taxonomic definition of species or genera for unicellular free-living algae (e.g., Ettl, 1983; Huber-Pestalozzi, 1961; Komárek and Fott, 1983; Starr, 1955). Ikeda and Takeda (1995) showed the species-specific differences of pyrenoids in some species of free-living and symbiotic *Chlorella*, and supported the separation of species of the genus into two groups; they concluded that the pyrenoid structure of *Chlorella* can be used as a taxonomic marker.

In many cases, the algae in symbiotic condition and cultured after isolation from the host show the same distinct pyrenoids surrounded by starch sheaths (e.g., Ikeda and Takeda, 1995; Reisser, 1975, 1976, 1988). We have much unpublished data that supports this previous work, when the algae had been isolated from their host from other sites by the same method as the present study and cultured in the same conditions. The algae observed in the present investigation, however, showed an alteration of the pyrenoid structure after being grown in the symbiotic and or in culture condition.

Reisser (1976) reported reduction of the starch deposition of symbiotic *Chlorella* in the presence of 3-(3,4-Dichlorophenyl)-1,1-dimethylurea (DCMU) or in darkness. Miyachi et al. (1986) reported effects of CO_2 concentration during growth on some species of *Chlorella* and *Scenedesmus*. Cells of some strains of *Chlorella vulgaris* and *Scenedesmus obliquus* under low CO_2 condition had a well-developed pyrenoid surrounded by starch, whereas those grown under high CO_2 condition had a less developed pyrenoid or no detectable pyrenoid. In the present observation, the unstable pyrenoids occurred without any of these manipulations but upon isolation from the host.

Although a loss of starch and a change of pyrenoid structure in degrading algae were observed in some previous works (e.g. Pocock 1955, Nozaki 1982, 1983, 1998), the different stages of degradation of the pyrenoids in the symbiotic alga are new observations. Also, the use of pyrenoid structures on symbiotic algae as taxonomical characters requires reconsideration on the treatment with circumspection.

Physiological features of symbiotic algae

The relationship between symbiotic algae and the host at the physiological and biochemical level are well known (e.g., Brown et al., 1974; Karakashian 1963; Muscatine et al., 1967; Reisser, 1976). Reisser (1984) clarified the physiological properties of symbiotic and free-living *Chlorella*. These results of the previous works showed that various symbiotic algae have different nutritional requirements. Loefer (1936) cultured the algae isolated from an aging *P. bursaria* on starch or nitrate agar medium, and reported that the colony obtained became colorless and finally died in culture. He also observed that the colorless but not yet dead colonies were able to recover their original green color and successively continued to further develop when they were transferred from the starch or nitrate agar medium to the medium with minerals (NaCl, $CaSO_4$, $MgSO_4$, KNO_3, $FeCl_3$). These facts suggest that the symbiotic algae can normally grow even on an inorganic medium. Two algal strains of different physiological properties were obtained from the algal colonies which were separated from a single ciliate and cultured on the CA medium, namely the strains surviving at least 20 months, and the other for only several months. This fact suggests that the strains with a short surviving term, at least, may be in a physiological state that depends on the host.

Further investigations should be extended to know if there are any differences of the pyrenoid morphology, the physiological features, and the position of both strains in SSU rDNA phylogenies.

Acknowledgements

We wish to express our special gratitude to Prof. J.D. Pickett-Heaps (The University of Melbourne, Australia) for kindly reading the manuscript and making helpful suggestions; Prof. J.M. Glime (Michigan Technological University, USA) and Prof. M. Galun (Editor; Tel Aviv University, Israel) for their great help in improvement of this manuscript, Prof. S. Watanabe (Toyama University, Japan), Prof. R.D. Seppelt (Australian Antarctic Division, Australia) and Dr. H. Nozaki (University of Tokyo, Japan) for their valuable comments and correction of the English text; and Dr. J. Lauer (Hiroshima University, Japan) for his critical reading of the earlier version of the manuscript. Financial support in the form of a Sasakawa Scientific Research Grant to M. Nakahara (12-218K) is gratefully acknowledged.

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