Significance of Photosynthetic Products of Symbiotic *Chlorella* to Establish the Endosymbiosis and to Express the Mating Reactivity Rhythm in *Paramecium bursaria*

Author(s): Miho Tanaka and Isoji Miwa
Source: Zoological Science, 13(5):685-692. 1996.
Published By: Zoological Society of Japan
DOI: 10.2108/zsj.13.685
URL: http://www.bioone.org/doi/full/10.2108/zsj.13.685

ZOOLOGICAL SCIENCE **13**: 685–692 (1996)

Significance of Photosynthetic Products of Symbiotic *Chlorella* to Establish the Endosymbiosis and to Express the Mating Reactivity Rhythm in *Paramecium bursaria*

Miho Tanaka and Isoji Miwa*

Department of Biology, Faculty of Science, Ibaraki University, Mito, Ibaraki 310, Japan

ABSTRACT—*Paramecium bursaria* contain several hundred cells of the green algae *Chlorella* as endosymbionts and are designated green. *Chlorella*-free white cells can be obtained from natural green cells by rapid growth in constant darkness (DD). *Chlorella* were isolated easily from their host cells and re-infected. The infection of *Chlorella* was restrained by a photosynthesis inhibitor (DCMU). This result can be related with the fact that symbiotic *Chlorella* release their photosynthetic products. Furthermore, when green cells were cultured in DD, the number of endosymbiotic *Chlorella* decreased and the density of host cells increased. On the other hand, the mating reactivity rhythm of green cells disappeared in DD. The photosynthetic products of symbiotic *Chlorella*, maltose and oxygen, induced the rhythms of their host cells in DD, but they could not shift the phase of the rhythms. Moreover, arrhythmic mutant white cells reverted to rhythmicity after infection with *Chlorella* isolated from wild type green cells. Thus the photosynthetic products released by endosymbiotic *Chlorella* have important roles in the establishing of the endosymbiosis and the expression of circadian rhythms in *P. bursaria* cells.

INTRODUCTION

Cells of the unicellular ciliate, *Paramecium bursaria*, contain several hundred green algae (Zoochlorella) established in the cytoplasm as endosymbionts (Loefer, 1936). *Chlorella*-free white cells can be obtained easily from natural green cells by rapid growth in constant darkness (DD). White cells also can be restored quickly to green ones by the infection of *Chlorella* isolated from green cells. The re-infection process starts with the induction of *Chlorella* to digestive vacuoles (DV) in the cytostom. Then *Chlorella* individually enter the perialgal vacuoles (PV). Since PV can not combine with the lysosome, *Chlorella* in the PV are not digested. When *Chlorella* stayed in DV, they are digested in due course (Meier and Wiessner, 1989). *P. bursaria* is a useful material for the study of the interaction between symbionts and the host cell. Cells of symbiotic *Chlorella* release their photosynthetic products, 90% of which is maltose and oxygen, and the host cells use them as living energy. Free-living *Chlorella* did not release their photosynthetic product of sugar, therefore the maltose release could be one of the essential factors in establishing symbionts (Weis, 1979, 1980).

On the other hand, the sexual interaction of *Paramecium* called mating reaction occurs upon mixing cells of complementary mating types when they are in the stationary phase and are sexually mature (Sonneborn, 1957; Hiwatashi, 1981). Furthermore, cells of *P. bursaria* exhibit high mating reactivity in the light phase and low in the dark phase of the light and dark cycle (LD 12:12 hr). After they are transferred to constant light (LL, 1,000 lux), they continue to show a clear circadian rhythm of mating reactivity for several days (Jennings, 1939; Miwa *et al.*, 1987). Circadian rhythms are shown in all levels of organisms including both eukaryotes and prokaryotes as basic behavior to adapt to global environments (Kondo *et al.*, 1993). Circadian rhythms are controlled by one or more internal oscillators often called a "Circadian clock". In spite of recent molecular genetic studies concerning circadian clocks (Aronson *et al.*, 1994; Kondo *et al.*, 1994; Sehgal, 1995), their mechanisms have not yet been made clear.

In this paper, we will report first the significance of photosynthetic products when *Chlorella* infect into *Paramecium* cells. We also show the disappearance of mating reactivity of green cells in DD is due to the digestion of the *Chlorella*. Furthermore, arrhythmic mutant cells are restored the mating reactivity rhythm by the infection of *Chlorella* isolated from wild type green cells.

MATERIALS AND METHODS

Strains and culture

Three strains of *Paramecium bursaria*, syngen 1 were used in this experiment: T316 (mating type IV, collected at Tsukuba in Ibaraki),

* To whom correspondence should be addressed.

Sj2 (mating type I, collected in Shimane), MC1w (mating type I, arrhythmic mutant strain). Strains T316 and Sj2 contain *Chlorella* symbionts and are thus designated green. Strains T316w and Sj2w are *Chlorella*-free white cells that have been induced from T316 and Sj2, respectively, by rapid growth in DD. Strain MC1w (white cells) was induced from Sj2w by treatment with MNNG (N-methyl-N'-nitro-N-nitrosoguanidine). Strain MCwT (green cells) was derived from MC1w by infection of *Chlorella* isolated from T316. All strains were cultured in a fresh lettuce juice medium, which had been inoculated with *Klebsiella pneumoniae* one day before use (Hiwatashi, 1968). Cultures were kept at 25°C under the light/dark cycle (LD 12:12 hr, 1,000 lux of cool-white fluorescent light) and transferred to constant light (LL) or dark (DD) condition in each experiment.

Test of mating reactivity

Mating reactivity of green cells was tested by mixing them with white "tester" cells of a complementary mating type. White cells were tested with green "tester" cells. Mating reactivity in cell populations was measured every 3 hr as follows: 10 cells were placed in each of 6 different wells of a depression glass plate, and about 100 highly reactive tester cells were added to each well. After 5 min, the percentage of mating reactive cells clumping with tester cells was counted.

To prepare tester cells with high reactivity, four groups of green and white cells were entrained to four light/dark cycles (LD 12:12 hr, staggered by 6 hr). Each group of tester cells was used twice in the mating reactivity test of consecutive 3-hr testing intervals. Since each group of tester cells showed high reactivity at least during 6 hr in a day, tester cells were always highly reactive in every test.

Treatment with maltose and oxygen

Mating reactivity rhythms of cells treated with maltose or oxygen were measured in DD. Cells entrained to a LD cycle (12:12 hr) were transferred to DD and added 10^{-2} M maltose or ventilated 10 ml oxygen. In this experiment, maltose or oxygen were added to the green cell cultures every 3 hr during 12 hr in three different schedules staggered by 6 hr as follows: A (12–24, 36–48, 60–72 hr); B (18–30, 42–54, 66–78 hr); C (24–36, 48–60 hr).

Re-infection of Chlorella

Chlorella were prepared for re-infection into the white cells as follows: Green cells in a 100 ml culture were concentrated by a hand centrifuge and then broken with an ultra-sonicator for 10 sec. *Chlorella* were collected through a 15 μm nylon mesh and suspended in K-DS solution (0.6 mM KH_2PO_4, 1.4 mM Na_2HPO_4, 2 mM $Na_3C_6H_5O_7$, 1.5 mM $CaCl_2$, pH 7.0). About 4×10^7 *Chlorella* cells/ml were mixed with about 2×10^3 white *Paramecium* cells. The number of *Chlorella* infected to the white cells was counted on slide glass under microscope. The green cells in this experiment were treated with 10^{-6} M photosynthesis inhibitor DCMU (Dichlorophenyl-Dimethylurea) for 6 hr.

RESULTS

Re-infection of Chlorella

Chlorella isolated from green cells can be infected into white cells and established as endosymbionts easily. To know the effects of photosynthetic products on the process of re-infection, *Chlorella* were isolated from green cells treated with DCMU. DCMU was added to green cell culture at the beginning of light phase in a LD cycle (12:12 hr). *Chlorella* were isolated from those green cells 6 hr after treatment of DCMU. Half of them were added to white cell suspension. The other half of isolated *Chlorella* were added to white cell suspension with maltose. Both of these cell suspensions were kept in K-DS solution containing DCMU in LL. As a control, *Chlorella* were isolated from untreated green cells and they were added to white cell suspension without DCMU. Five white cells were isolated randomly every time and the number of *Chlorella* in the cells was counted. As seen in Fig.1, the control *Chlorella* increased rapidly until 6 hr after infection and then they became stable. On the other hand, *Chlorella* treated with DCMU only were hard to be infected to white cells. They were less than half of the control even at the maximum values, and then they

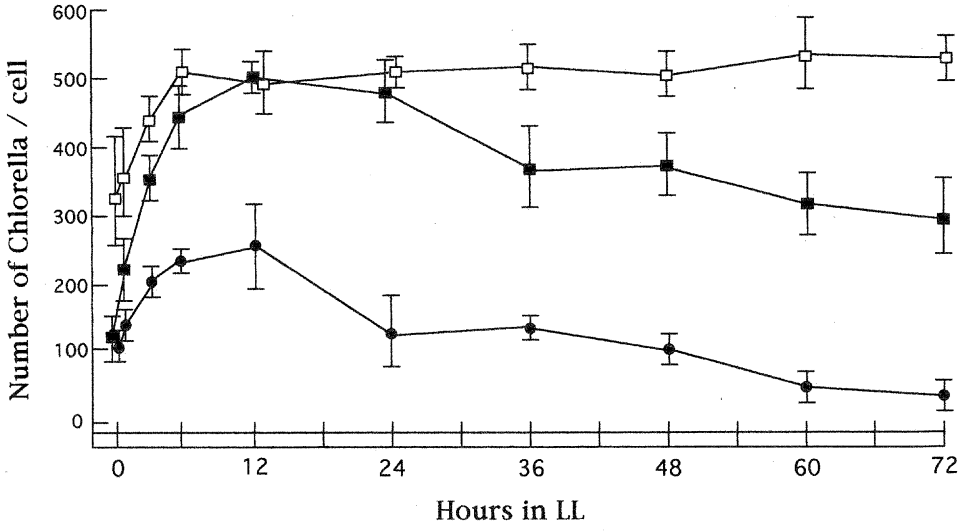

Fig. 1. The effects of DCMU and maltose on the re-infection of *Chlorella*. *Chlorella* were isolated from green cells (T316) that were treated with 10^{-6} M DCMU for 6 hr, and they were added to white cell (T316w) suspension containing DCMU in absence (●) and presence (■) of maltose in LL. Open square (□) shows untreated *Chlorella* as a control. Abscissa indicates the time after adding of *Chlorella* to white cell suspension. The bar at each point indicates SD of results from 5 times measurements.

decreased gradually. However, *Chlorella* increased to control level in presence of maltose 12 hr after addition of *Chlorella*. After that, they began to decrease and they could not be stable.

Chlorella *were digested by the host cells in DD*

To know the necessity of photosynthesis of *Chlorella* for maintain the symbiont in the host cell, the transition of the number of *Chlorella* in green cell was examined following the time in DD. *Chlorella* began to decrease after 6 hr in DD and 70% of *Chlorella* were lost after 60 hr (Fig. 2). Moreover, the cell densities of green and white *Paramecium* cultures were measured in DD. As seen in Fig. 2, green cells increased in inverse proportion to the decrease in symbiotic *Chlorella*. White cells showed almost the same density for 3 days. All cells were not given culture medium during this experiment. Since the decrease ratio of number of *Chlorella* was larger than the increase ratio of number of green cells, it was considered that some symbiotic *Chlorella* were digested by host cells, and green cells divided in DD.

Green cells did not show a mating reactivity rhythm in DD

Green cells of *P. bursaria* show mating reactivity in the light period, but not in the dark period, when exposed to a light/dark cycle (LD 12:12 hr). After they were transferred to constant-light (LL) conditions, they continued to show a circadian rhythm of mating reactivity. What is the mating reactivity rhythm of green cells in DD, in which green cells divided as shown in Fig. 2? Mating reactivity rhythms of green cells and white cells in two natural strains (T316 and Sj2) were measured in LD, LL and DD. Results are shown in Fig. 3 and Fig. 4. Mating reactivity of green cells and white cells in both strains were entrained to a LD 12:12 hr cycle and their rhythms persisted in LL. But mating reactivity of green cells appeared different to the ones of white cells in DD. In strain T316, although white cells (T316w) showed the same rhythm in DD as in LL, green cells hardly exhibited any mating reactivity (Fig. 3). In strain Sj2, white cells (Sj2w) kept a high mating reactivity of more than 80% in DD, whereas green cells showed low mating reactivity in DD, about 20% (Fig. 4). These results indicate that symbiotic *Chlorella* inhibit the expression of the mating reactivity of *Paramecium* cells in DD.

The effects of maltose and oxygen on the expression of mating reactivity rhythm

Symbiotic *Chlorella* are doing photosynthesis and their products, maltose and oxygen, are released inside the *Paramecium* cell (Weis, 1979). It is expected that the lack of these products in DD cause to the disappearance or decline of the mating reactivity of green cells as shown in Fig. 3 and Fig. 4. Then the effects of maltose and oxygen on the expression of mating reactivity were examined in DD. In this experiment, maltose or oxygen were added to green cell cultures in DD every 3 hr in three different schedules as shown in Methods. In each case, green cells showed mating reactivity in the subjective day phase in spite of the treatments of maltose and oxygen at different phases (Fig. 5). Thus the maltose and oxygen forced these cells to express the mating reactivity rhythms in DD, but not to shift the phases of these rhythms.

Chlorella *rescued the mating reactivity rhythm in arrhythmic mutant*

One arrhythmic mutant strain MC1w was induced from Sj2w by treatment with nitrosoguanidine (Miwa *et al.*, 1996a). As shown in Fig. 4, Sj2w appeared arrhythmicity in DD only. MC1w expressed high mating reactivity continuously not only in DD but also in LD and LL. These white cells (MC1w) were

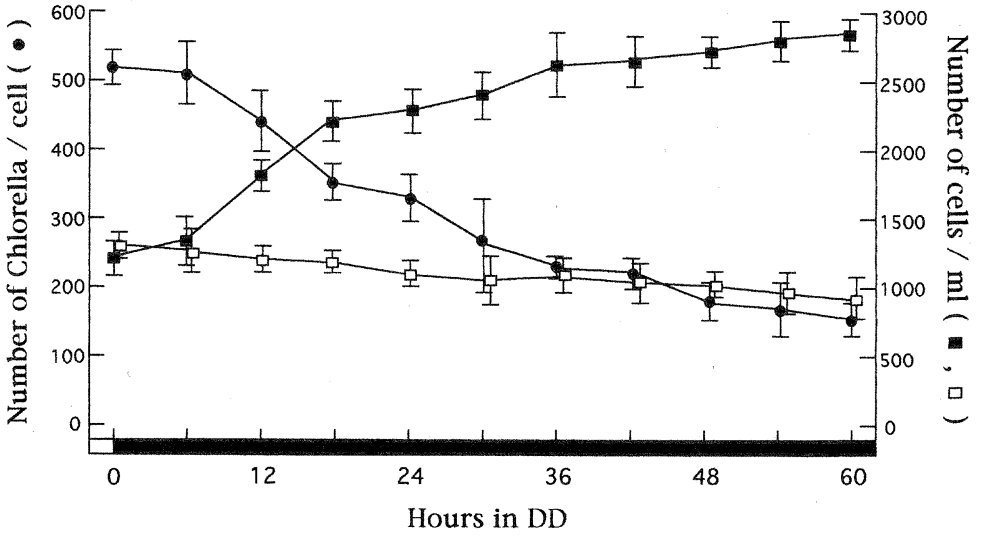

Fig. 2. The transition of densities of symbiotic *Chlorella* and host cells in DD. The number of *Chlorella* (●) in a green cell (T316) was counted each time in DD. The densities of green cells (■) and white cells (□) in experimental cultures were measured every 6 hr in DD as follows: 1 ml of cell suspension was putted in a depression glass plate from each cultures. They were added 2 mM $NiCl_2$ to stop the swimming, and the number of cells was counted. The bar at each point indicates SD.

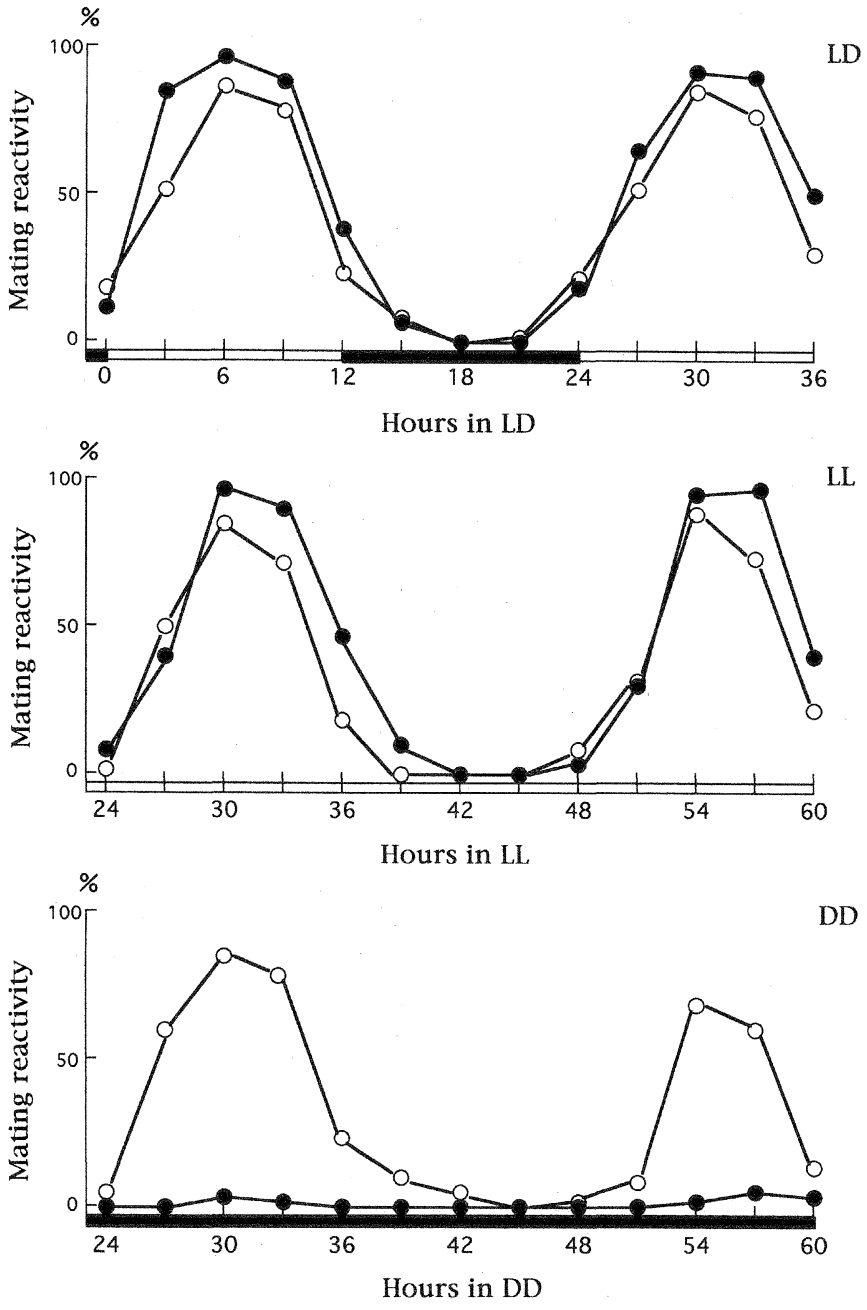

Fig. 3. Mating reactivity rhythms of natural strain T316. Mating reactivity of green cells (●) and white cells (○) was tested in LD, LL and DD every 3 hr by mixing them with "tester" cells of a different color and of a complementary mating type. Green cells showed no mating reactivity in DD.

infected with *Chlorella* isolated from T316 and the mating reactivity of the green cells (MCwT) was measured in LD, LL and DD. As seen in Fig. 6, MC1w had kept high mating reactivity continually in each condition. On the other hand, green cells (MCwT) induced from MC1w showed rhythmic mating reactivity in LD and LL. But they did not show the rhythmicity in DD and their reactivity was less than 50 %. It seems that the photosynthetic products of symbiotic *Chlorella* had reverted the arrhythmic mutant to mating reactivity rhythms in LD and LL.

DISCUSSION

Unicellular ciliate, *Paramecium bursaria* is an interesting material as a model of a coexisting plant cell in a single animal cell. Endosymbiotic *Chlorella* release their photosynthetic products, maltose and oxygen into their host cells. Especially the maltose release is a feature of the *Chlorella* symbiont in the cells of *P. bursaria*. It has been reported that *Chlorella* released glucose could not be re-infected into the cells of *P. bursaria* (Weis, 1980). It is considered that maltose is a signal

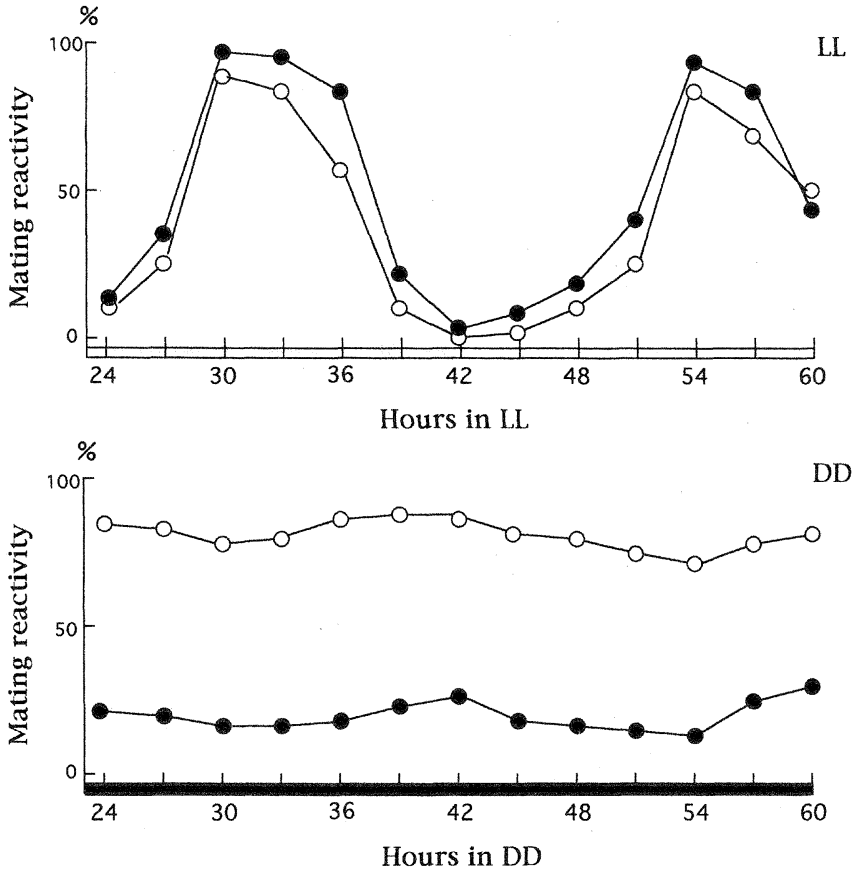

Fig. 4. Mating reactivity rhythms of strain Sj2. Mating reactivity of green cells (●) and white cells (○) was tested in LL and DD. In DD, white cells showed arrhythmic high mating reactivity continuously, and green cells expressed arrhythmic low mating reactivity.

for infecting to *Paramecium* cells. In this study, *Chlorella* treated with photosynthesis inhibitor DCMU were tested for their infectiousness. As seen in Fig. 1, they were hardly infected to *Paramecium* cells. Even if many *Chlorella* were induced into the host cells at first, they could not establish themselves as a symbiont. Their final infectiousness was less than 50 algal cells/one *Paramecium* cell. On the other hand, when *Chlorella* added to white cell suspension with maltose, they were infected to white cells as the same amount as ones untreated with DCMU. But they began to decrease 12 hr after infection and they could not be stable. These results suggest that maltose release from symbiotic *Chlorella* has important roles on the process of re-infection of *Chlorella*. First it would function as one of triggers to induce the *Chlorella* into digestive vacuoles (DV). And then, maltose may be required when the *Chlorella* enters the perialgal vacuoles (PV) to establish a symbiont. It seems that when *Chlorella* were inhibited a release of their own photosynthetic products in *Paramecium* cells by DCMU, they could not enter the PV and were digested in the DV.

On the other hand, photosynthetic products are also necessary to keep *Chlorella* as the symbionts in the *Paramecium* cell. In DD, the number of *Chlorella* in the green cell decreased, and reversely, the density of host cells in the culture increased in the progress (Fig. 2). It was expected that these relationships were caused by the lack of photosynthetic products released from *Chlorella*. Cells of *P. bursaria* would digest the endosymbiotic *Chlorella* that could not photosynthesize in DD. *Paramecium* cells can not express generally their mating reactivity in growth phase. Since the cell cycle of green cells was proceeding in DD, the mating ability of green cells disappeared or declined as seen in Figs. 3 and 4. When green cells were given maltose or oxygen in DD, they could show the mating reactivity rhythms (Fig. 5). Therefore the photosynthetic products of *Chlorella* were necessary to express the mating reactivity of green *Paramecium* cells. To make sure of these assumptions, we tried to assay the mating reactivity of cells treated with the photosynthesis inhibitor DCMU in LL. However, not only the green cells, but also the white cells never expressed any mating reactivity under DCMU (data not shown). Probably, DCMU has the effect to inactivate the mating reaction itself. Thus we should try to use other photosynthesis inhibitors that do not affect to mating reactivity of *Paramecium* cells.

Next, the effects of photosynthetic products of *Chlorella* on the expression of mating reactivity rhythms of *P. bursaria* were considered. Since *P. bursaria* show many kinds of circadian behaviors including mating reactivity and photo-

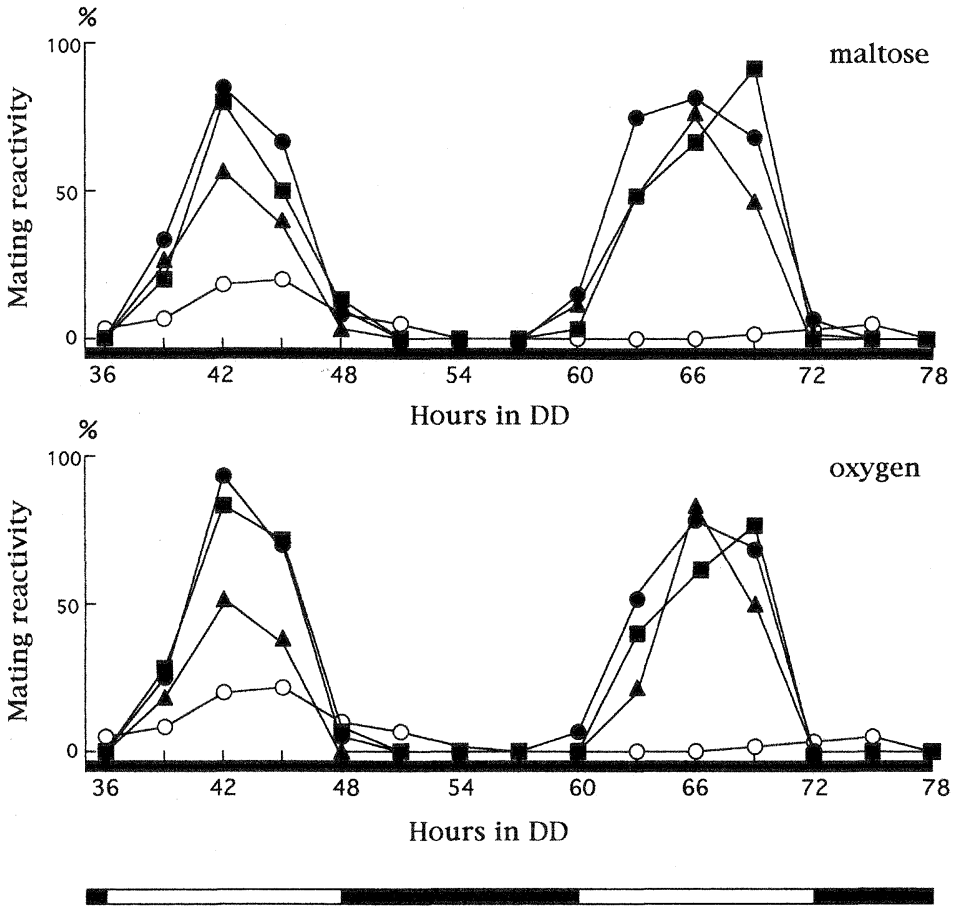

Fig. 5. Mating reactivity rhythms of green cells (T316) treated with maltose or oxygen in DD. Cells entrained to a LD cycle (12:12 hr) were transferred to DD and added 10^{-2} M maltose or ventilated 10 ml oxygen every 3 hr during 12 hr in three different schedules (A-C) staggered by 6 hr as follows: A (12-24, 36-48, 60-72 hr) (●); B (18-30, 42-54, 66-78 hr) (▲); C (24-36, 48-60 hr) (■). Open circle (○) shows a control of untreated cells. Abscissa indicates the time after transference to DD. Lower bar indicates subjective day and night phases. Mating reactivity appeared in subjective day phase in spite of the treatment of maltose and oxygen at different phases.

accumulation, they are suitable materials to analyze the rhythms at cellular level (Miwa *et al.*, 1987; Johnson *et al.*, 1989). In a previous paper (Miwa *et al.*, 1996b), we reported that symbiotic *Chlorella* forced the host cells to shift the phase and lengthen the period of photoaccumulation rhythm in LL. In this study, several effects of symbiotic *Chlorella* were also observed on the mating reactivity rhythms. As described in Results, green cells containing *Chlorella* did not exhibit mating reactivity in DD. Whereas, green cells given maltose or oxygen showed a mating reactivity rhythm in DD similar to LD and LL (Fig. 5). Moreover, arrhythmic mutant cells (MC1w) had been restored circadian rhythmicity in LD and LL by the infection of *Chlorella*, but not in DD (Fig. 6). These results also indicate that the photosynthesis of symbiotic *Chlorella* induce the mating reactivity rhythms. Moreover, when green cells were given maltose or oxygen at different three phases staggered by 6 h in DD, they showed mating reactivity rhythms of the same pattern. Photosynthetic products seem to act to express the mating reactivity according to the clock of host cell, not to express the mating reactivity directory. It will be of interest to examine the mating reactivity rhythms of MC1w after infection of *Chlorella* entrained with different LD cycles.

Then, how act *Chlorella* on host cells? Although cells of MC1w are mutants that express arrhythmic mating reactivity continuously, they show a circadian rhythm of photoaccumulation (Miwa *et al.*, 1996a). Therefore their circadian system is oscillating normally if these two rhythms are operated by the same oscillator. In addition, it is expected that the arrhythmicity of mating reactivity is attributed to mitochondrial mutation because this property of MC1w is inherited cytoplasmically (Miwa *et al.*, 1996a). If this is true, it is suggested that the mitochondria play an important part in the expression process of circadian mating reactivity rhythm. Furthermore, symbiotic *Chlorella* might interact with the host cells through the mitochondria. Further experiments and consideration are needed on this point of view.

REFERENCES

Aronson BD, Johnson KA, Loros JJ, Dunlap JC (1994) Negative feedback defining a circadian clock: autoregulation of the clock gene *frequency*. Science 263: 1578–1584

Hiwatashi K (1968) Determination and inheritance of mating type in

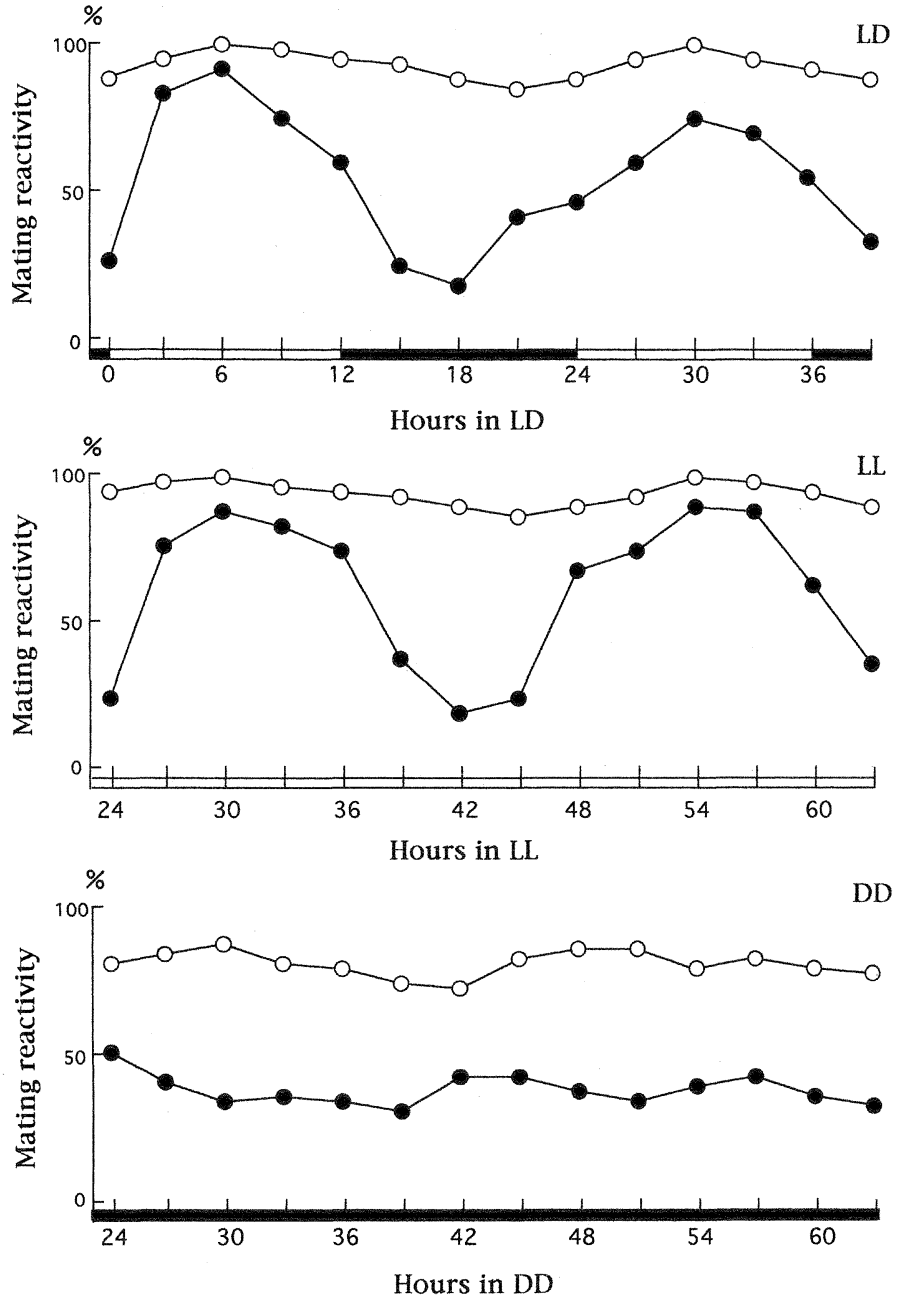

Fig. 6. Mating reactivity of arrhythmic mutant cells, MC1w (○), and the re-infected cells with *Chlorella* isolated from T316, MCwT (●). The infected *Chlorella* forced the arrhythmic mutant cells to revert the mating reactivity rhythms in LD and LL, but not in DD.

Paramecium caudatum. Genetics 58: 373–386

Hiwatashi K (1981) Sexual interactions of the cell surface in *Paramecium*. In "Sexual Interactions in Eukaryotic Microbes" Ed by DH O'Day, PA Horgen, Academic Press, New York, pp 351–378

Jennings HS (1939) Genetics of *Paramecium bursaria*: I. Mating type and groups, their interrelations and distribution; mating behavior and self sterility. Genetics 24: 202–233

Johnson CH, Miwa I, Kondo T, Hastings JW (1989) Circadian rhythm of photoaccumulation in *Paramecium bursaria*. J Biol Rhythms 4: 405–415

Kondo T, Strayer CA, Kulkarni RD, Taylor W, Ishiura I, Golden SS, Johnson CH (1993) Circadian rhythms in prokaryotes: luciferase as a reporter of circadian gene expression in cyanobacteria. Proc Natl Acad Sci 90: 5672–5676

Kondo T, Tsinoremas NF, Golden SS, Johnson CH, Kutsuna S, Ishiura M (1994) Circadian clock mutant of cyanobacteria. Science 266: 1233–1236

Loefer JB (1936) Isolation and growth characteristics of the "Zoochlorella" of *Paramecium bursaria*. Am Midl Nat 70: 184–188

Meier R, Wiessner W (1989) Infection of algae-free *Paramecium bursaria* with symbiotic *Chlorella* sp. isolated from green *Paramecia*. J Cell Sci 93: 571–579

Miwa I, Nagatoshi H, Horie T (1987) Circadian rhythmicity within single cell of *Paramecium bursaria*. J Biol Rhythms 2: 57–64

Miwa I, Izumo T, Sonoda T (1996a) Cytoplasm rescues an arrhythmic mutant on the circadian rhythm of mating reactivity in *Paramecium bursaria*. J Euk Miclobiol 43: 231–236

Miwa I, Fujimori N, Tanaka M (1996b) Effects of symbiotic Chlorella on the period length and the phase shift of circadian rhythms in *Paramecium bursaria*. Europ J Protistol, in press

Sehgal A (1995) Genetic dissection of the circadian clock: a *timeless* story. Neuroscience 7: 27–35

Sonneborn TM (1957) Breeding systems, reproductive methods and species problems in Protozoa. In "The Species Problem" Ed by E Mayr, Washington DC, Am Assoc Adv Sci, pp 155–324

Weis DS (1979) Correlation of sugar release and Concanavalin A agglutinability with infectivity of symbiotic algae from *Paramecium bursaria* for aposymbiotic *P. bursaria*. J Protozool 26: 117–119

Weis DS (1980) Free maltose and algal cell surface sugars are signals in the infection of Paramecium bursaria by algae. In "Endocytobiology Vol 1" Ed by W Schwemmler, HEA Schenck, Walter de Gruyter & Co, Berlin-New York, pp 105–112

(Received May 13, 1996 / Accepted June 13, 1996)