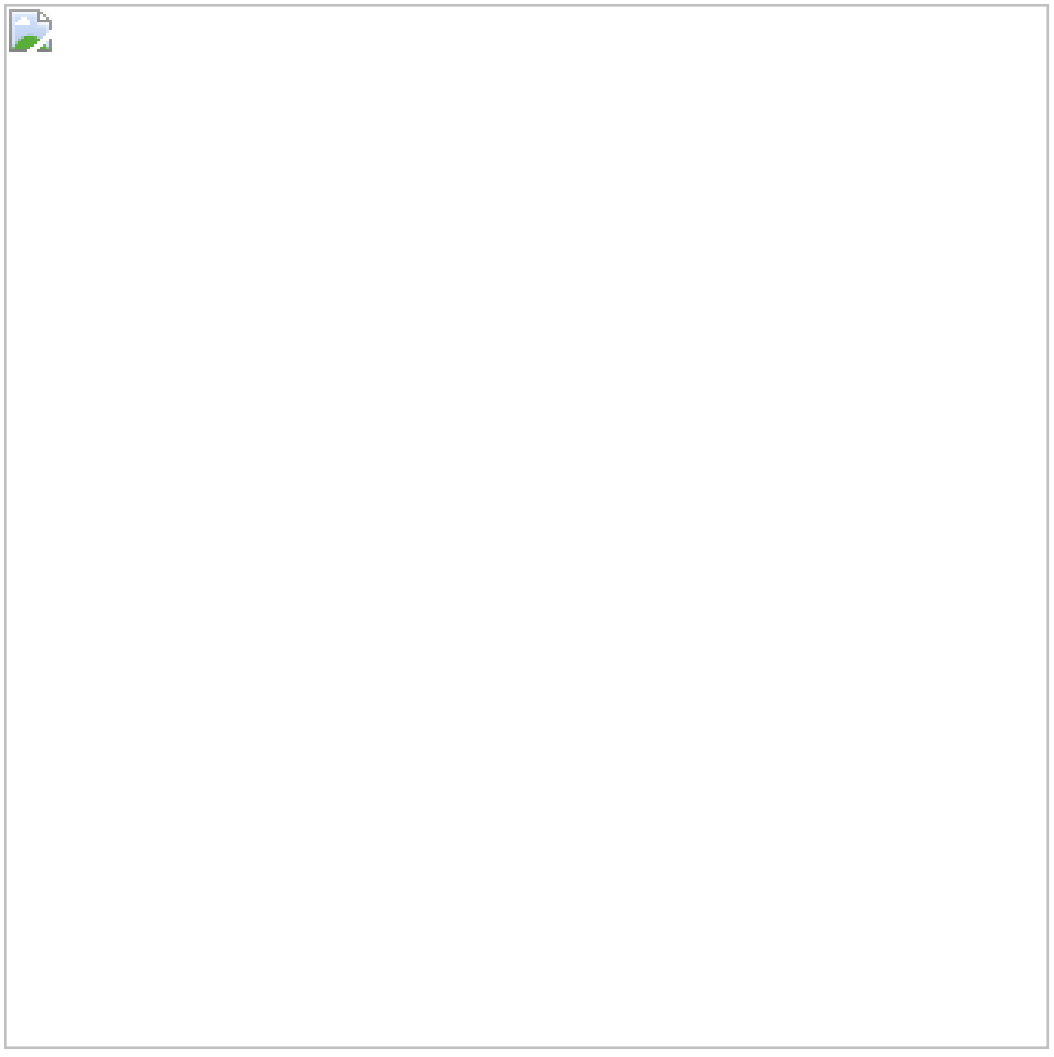

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Encystment-Inducing Factors in the Ciliate *Euplotes elegans*

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ABSTRACT—Although starvation is considered one of the most important induces of ciliate encystment, its nature has been unclear. *Euplotes* is a well-known ciliate genus, but the relationship in *Euplotes* between encystment and food has not been reported. The encystment of *Euplotes elegans* is facilitated when it is transferred to Chalkley's solution without bacteria as food. A higher ciliate density also facilitates encystment. Thus, starvation and ciliate density needed to be examined. Ciliates were inoculated into 3 treatments: Chalkley's solution with formaldehyde-fixed bacteria as nutritive particles (FFB group), with polystyrene latex particles as non-nutritive particles (PLP group), and without particles (control group). Cysts appeared fastest and ciliate numbers increased in the FFB group. Although the encystment kinetics of the PLP group was similar to that of the control group, the encystment rate of the PLP group was lower than that of the control group in the earliest phase. This suggests that the ciliates were temporarily deceived into feeding on PLP, because they had food vacuoles containing PLP during the earliest phase of incubation. A cell-free old culture solution from a stationary phase, which probably contained excreted substances from high-density ciliates, also facilitated encystment.

Key words: ciliate, encystment, food

INTRODUCTION

Encystment is a well-known phenomenon in many species of protozoa and has been investigated for a long time. In recent years, however, few papers have dealt with causes of encystment, even though such research was in vogue until the 1950s (van Wagtendonk, 1955; Corliss and Esser, 1974).

Resent papers on ciliate encystment have concentrated on the cyst wall structures observed under transmission and scanning electron microscopy (e.g. Martín-González *et al*., 1994) and on the chemical composition of the cyst wall as analyzed by biochemical (e.g. Calvo and de Miguel, 1995-96) or cytochemical methods (e.g. Izquierdo *et al*, 2000).

Earlier studies reported that factors inducing encystment included starvation, excess food, salt concentration, cell density, and accumulation of metabolites (van Wagtendonk, 1955; Corliss and Esser, 1974). The latest papers on the subject are those of Yonezawa (1986b) and Dallai *et al*. (1985), who reported exogenous factors inducing encystment in the genus *Euplotes*.

Of the 51 known *Euplotes* species (Curds, 1975), 13 are capable of encystment (Rawlinson and Gates, 1985). Exogenous induction of encystment has been reported in only 5. Encystment in 4 marine species-*E. taylori* (Garnjobst, 1928), *E. harpa* (Mélant, 1922), *E. longipes* (Klee, 1926), and *E. rariseta* (Dallai *et al*., 1985)-was induced by increasing the salt concentration. Encystment in a freshwater species, *E. encysticus*, was induced by culture in a cell-free medium (Yonezawa, 1986b). Although starvation is considered the most important inducer of ciliate encystment (Gutiérrez *et al*., 1990), the relationship between starvation and encystment in the genus *Euplotes* has not been reported. I examined effect of food or ciliate density on encystment in *E. elegans* (Fig. 1). It was clear that encystment was facilitated in cell-free old culture solution (CFS) without food condition, and was delayed to be fed non-nutritive particles.

MATERIALS AND METHODS

Culture

Euplotes elegans was isolated from wastewater in Nara, Japan, in May 1994. The culture medium (CM) was 0.1% liquid New Calorie Mate (Otsuka Seiyaku Co.) in Chalkley's solution (0.1% NaCl, 0.004% KCl, 0.006% $CaCl_2$). The ciliates were inocu-

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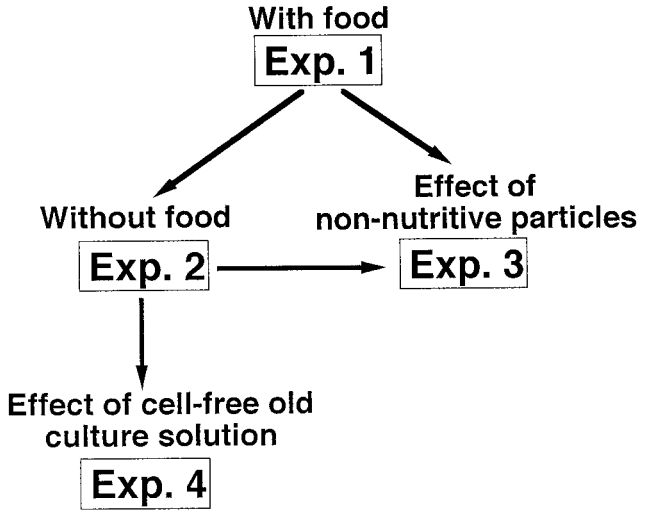

Fig. 1. Scheme of experiments.

lated into CM in which the bacteria *Klebsiella pneumoniae* had reached a stationary phase. Aliquots (18 ml each) of the cell suspension were put into 9-cm plastic Petri dishes. Before the experiments were carried out, the ciliates were collected by centrifugation for 3 min at ca 50g and washed 3 times with Chalkley's solution.

Experiments

Fig. 1 summarizes these experiments procedure. Experiments were carried out in 3-cm plastic Petri dishes containing 2 ml of the washed ciliates in CM (Experiment [Exp.] 1) or Chalkley's solution (Exp. 2 to 4) at 25±1°C.

Exp. 1: Effect of bacterial and ciliate densities on encystment

Ciliates were inoculated into CM containing bacteria at a stationary phase (ca. 6.3×10^5 to 1.1×10^6 cells ml^{-1}) then incubated in Petri dishes at 4 densities: 200 (CM200), 1000 (CM1000), 2000 (CM2000), or 5000 (CM5000) cells ml^{-1}. For each density, the ciliates in 3 Petri dishes and the bacteria in another 3 Petri dishes were counted every 12 hr from day 1 through day 5, and every 24 hr from day 6 through day 10. The ciliates were fixed in saturated picric acid solution and counted under a light microscope. Cell suspensions of the bacteria were filtered through Nucleopore membranes (pore size 3 μm), and the cells were counted by an optical density method using a double-beam spectrophotometer at 550 nm (Shimadzu, model UV-140-01) and by a colony-counting method. The medium used for the colony-counting method contained 5 g proteose peptone, 0.5 g yeast extract, 2.25 g KH_2PO_4, 1.5 g $K_2HPO_4{\bullet}12H_2O$, and 20 g agar per 1000 ml.

Exp. 2: Effect of ciliate density on encystment in the absence of food

Washed ciliates (density, 200 [C200], 1000 [C1000], or 2000 [C2000] cells ml^{-1}) were inoculated into Chalkley's solution without bacteria. Sampling methods, counting intervals, and counting methods were as in Exp. 1.

Exp. 3: Effects of nutritive and non-nutritive particles on encystment

The experimental solutions were bacteria-free Chalkley's solution (control), Chalkley's solution with polystyrene latex particles (PLP; diameter ca. 1 μm; non-nutritive), and Chalkley's solution with formaldehyde-fixed bacteria (FFB; nutritive) (Sugai, 1989). The PLP and FFB suspensions were prepared to the same absorbance (OD_{550} = 0.1). To minimize the accumulation of substances excreted from the ciliates, the ciliate density was 100 cells ml^{-1}. The cysts were counted without fixation under light microscopy every 12 hr for 5 days.

Exp. 4: Effect of cell-free ciliate culture conditioned medium (CFM) on encystment

The experimental solutions were a cell-free ciliate culture conditioned medium (CFM), 1/10 CFM diluted with Chalkley's solution, and Chalkley's solution. CFM was prepared by filtering a 2-day-old ciliate suspension in Chalkley's solution (several hundred cells ml^{-1}) through a 3-μm Nucleopore membrane filter. To minimize the accumulation of substances excreted from the ciliates, the ciliate density was 20 cells ml^{-1}. Cysts were counted as in Exp. 3.

Statistical analysis

In all cases, the degree of the encystment was expressed as percentage of the total number of ciliate (= No. of cysts × 100 / No. of total ciliates (%)) and arcsine-transformed before statistical analysis. In all tests , the significance level was α=0.05.

In all experiments and for comparison between the CM and C series, a two-way analysis of variance using model I was completed, with initial ciliate density (Exps. 1 and 2) or treatment (Exps. 3 and 4) and time as factors. In Exp. 3, the comparison between the control and PLP groups at each time point was analyzed further by using the Mann-Whitney U-test.

df, SS, F and NS referred to degree of freedom, sum of suquare, F value and no significance.

RESULTS

Exp. 1: Effects of bacterial and ciliate densities on encystment

Starting densities were 200, 1000, 2000, and 5000 ciliates ml^{-1}. Because the bacteria were in the stationary phase, the numbers of bacteria available as food were ca. 3200, 760, 510, and 160 per ciliate, respectively. The ciliate densities in the CM200 and CM1000 plates increased during the early phase after inoculation. The densities in the CM2000 plates did not change, whereas those in the CM5000 plates decreased gradually after inoculation. The higher the ciliate density was, the faster cysts appeared (4 days in CM200, 3 in CM1000, 2 in CM2000, and 2 in CM5000) (Fig. 2). Dividing ciliates were observed for 4 days in CM200 and 3 days in CM1000, but did not occur after the appearance of cysts. The differences in encystment rates were significant ($df_{density}$=3, df_{time}=10; $SS_{density}$=44039.8, SS_{time}=96488.9; $F_{density}$=1059.9, F_{time}=419.7; both $p < 0.01$, $SS_{interaction}$=23969.1, $F_{interaction}$=34.8, $p < 0.01$). The number of ciliates and the number of bacteria per ciliate both affected the lag time before encystment started, although there was significant interaction between these parameters.

Exp. 2: Effect of ciliate density on encystment in the absence of food

Starting ciliate densities were 200, 1000, and 2000 ciliates ml^{-1}. Cysts were observed after 2 days in C200 and after 1 day in C1000 and C2000. The differences in encystment rates were significant ($df_{density}$=2, df_{time}=15; $SS_{density}$=5140.4, SS_{time}=128583.9, $F_{density}$=61.9, F_{time}=100.3, both $p < 0.01$ for both parameters, $SS_{interaction}$=2977.9, $F_{interaction}$=1.2, NS). The total number of vegetative ciliates decreased throughout the observation period at all densities (Fig. 3).

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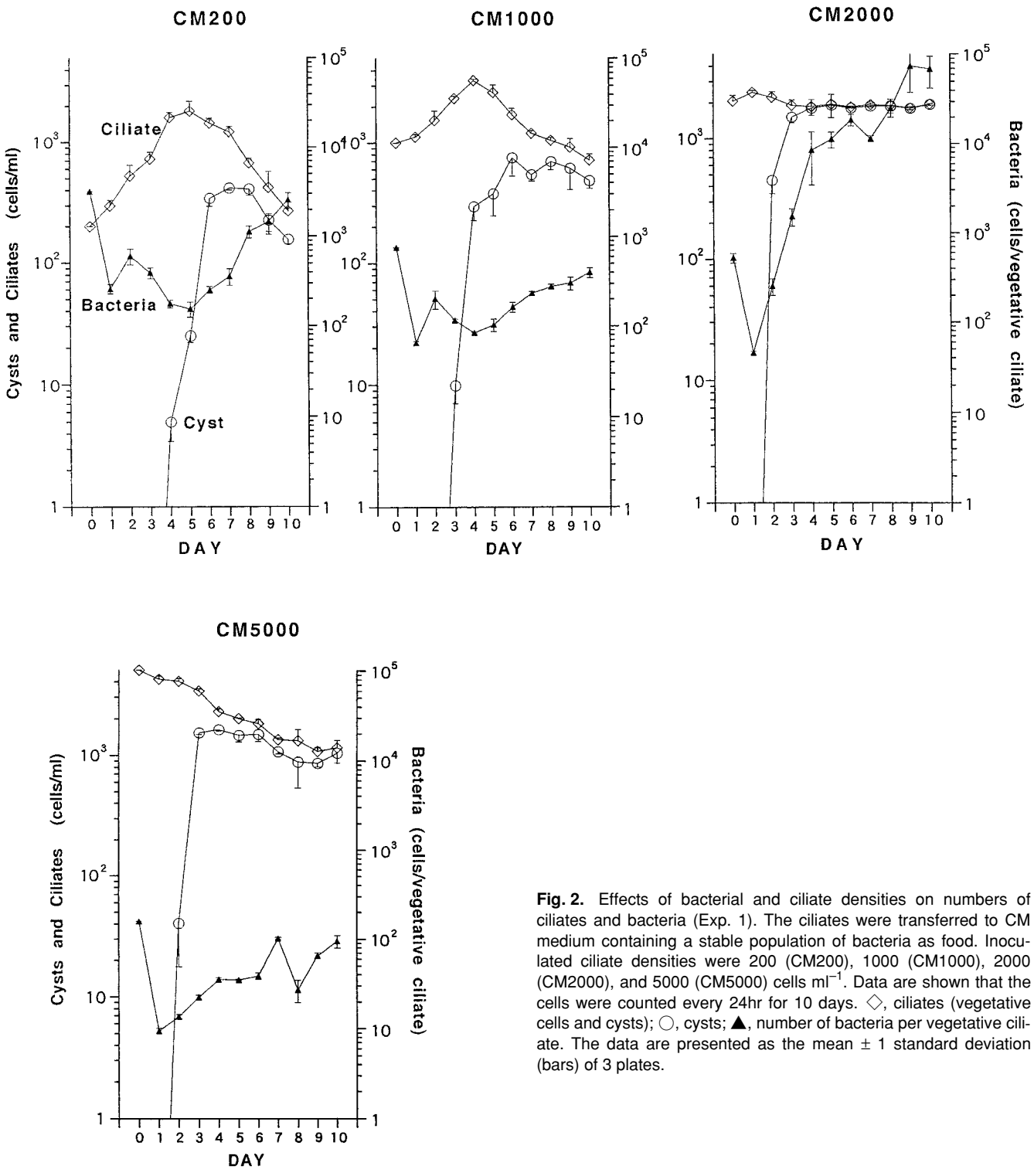

Fig. 2. Effects of bacterial and ciliate densities on numbers of ciliates and bacteria (Exp. 1). The ciliates were transferred to CM medium containing a stable population of bacteria as food. Inoculated ciliate densities were 200 (CM200), 1000 (CM1000), 2000 (CM2000), and 5000 (CM5000) cells ml^{-1}. Data are shown that the cells were counted every 24hr for 10 days. ◇, ciliates (vegetative cells and cysts); ○, cysts; ▲, number of bacteria per vegetative ciliate. The data are presented as the mean ± 1 standard deviation (bars) of 3 plates.

Exp. 3: Effect of nutritive and non-nutritive particles on encystment

The kinetics of cyst numbers were similar between the PLP and control groups but significantly different between the control and FFB groups ($df_{treatment}=2$, $df_{time}=10$; $SS_{treatment}=71801.7$, $SS_{time}=56999.7$; $F_{treatment}=68.0$, $F_{time}=100.9$; $p < 0.01$ for both parameters; $SS_{interaction}=18982.3$, $F_{interaction}=16.8$, $p < 0.01$). Because the ciliates had multiplied, the number of cysts in the FFB group was higher than those in the other groups after 3 days. At days 0.5 and 1.5 after ciliate inoculation, the number of cysts in the PLP group was significantly less than that in the control group (Mann-Whitney U-test; $z=-2.0$ for both days, $p < 0.05$ for both days). During the early phase, the ciliates in the PLP group had

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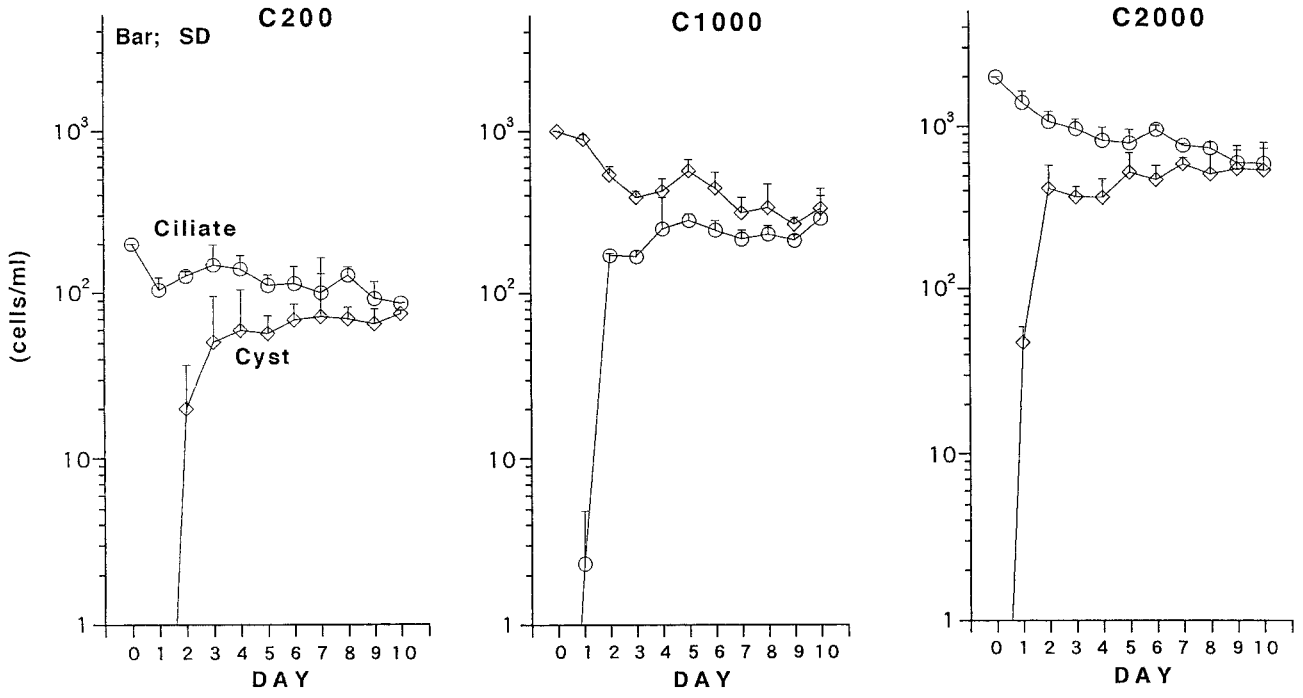

Fig. 3. Effect of ciliate density on encystment in the absence of food (Exp. 2). The ciliates were transferred to Chalkley's solution, which had a low bacterial density (10.1 cells ml^{-1}). Ciliates could not grow in this solution. Inoculated ciliate densities were 200 (C200), 1000 (C1000), and 2000 (CM2000) cells ml^{-1}. Data are shown that the ciliates were counted every 24hr for 10 days. ◇, ciliates; ○, cysts. The data are presented as the mean ± 1 standard deviation (bars) of 3 plates.

food vacuoles containing PLP.

Exp. 4: Effect of cell-free ciliate culture conditioned medium (CFM) on encystment

The lag time after inoculation until cyst appearance was longer for the control group than for the 1/10 CFM group, whose lag time was longer than CFM group ($df_{treatment}=2$, $df_{time}=10$; $SS_{treatment}=13901.5$, $SS_{time}=54132.6$; $F_{treatment}=9.7$, $F_{time}=34.1$; $p < 0.01$ for both parameters, $SS_{interaction}=10025.3$, $F_{interaction}=3.2$, $p < 0.01$).

DISCUSSION

Comparison of the results obtained from Exp. 1 (with bacteria) (Fig. 2) and Exp. 2 (without bacteria) (Fig. 3) showed that the higher the ciliate density was, the faster the cysts appeared. In all density groups, the numbers of bacteria per mL decreased on day 1, then increased and became stable after day 2. This result suggests that the quantity of food per vegetative ciliate affected the numbers of ciliates and bacteria (Fig. 2). To my knowledge, the numbers of bacteria and ciliates had not previously been counted during the same experiment assessing encystment. The data in Fig. 2 suggest that a bacterial density of $\leq 10^2$ cells per vegetative ciliate may not support the ciliates. Cysts appeared fastest in the C2000 plates, and the encystment rate was highest in the CM2000 plates. These results suggest that the food and ciliate density present under CM2000 and C2000 conditions was most favorable for encystment.

One of my aims was to clarify the meaning of the term "starvation" with respect to inducing encystment. Does it mean having nothing to eat, or having something to eat but not enough to support proliferation? Most of the earlier experiments concerning the effects of starvation inoculated ciliates into a solution without bacteria (van Wagtendonk, 1955; Corliss and Esser, 1974; Gutiérrez *et al.*, 1990). I conducted Exp. 3 (Fig. 4) to clarify this question. A reduced cil-

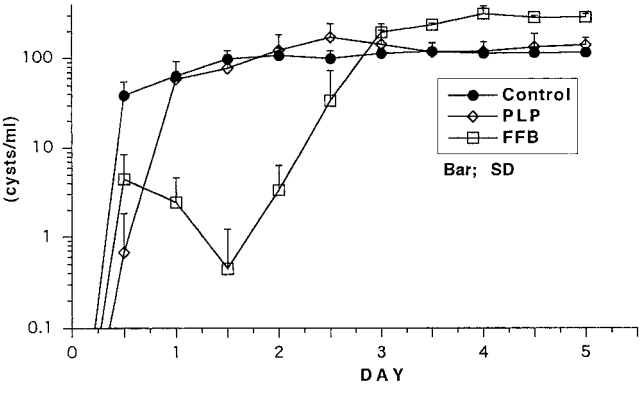

Fig. 4. Effect of nutritive and non-nutritive particles on encystment (Exp. 3). The ciliates were transferred to bacteria-free Chalkley's solution (control) , Chalkley's solution with polystyrene latex particles (PLP), or Chalkley's solution with formalin-fixed bacteria (FFB). Cysts were counted every 12 hr for 5 days. ●, control group; ○, PLP group; □, FFB group. The data are presented as the mean ± 1 standard deviation (bars) of 3 plates.

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iate density of 100 ciliates ml^{-1} and killed bacteria as the nutrition source (FFB) were used to minimize the accumulation of excretory substances from living ciliates and bacteria. Polystyrene latex particles (PLP) of similar size to bacteria were used as a non-nutritive imitation food. Cysts appeared later in the FFB group than in the control and PLP groups. The delay may have come from the fact that the ciliates fed on FFB and proliferated. The encystment rate of the PLP group was low during the earliest phase (to 1.5 day), although the overall encystment kinetics of the PLP group was similar to those of the control group (Fig. 4). Feeding on PLP may have caused the low initial encystment rate in the PLP group, because the ciliates contained PLP in their food vacuoles. I think that the ciliates were deceived temporarily and did not encyst. In other words, a delayed in stopping the cell cycle was delayed beginning of encystment. For such leading to my speculation, Yonezawa (1985) was reported that in *E. encysticus*, the encystment rate decreased in advanced changes in the cell cycle.

An increased ciliate density results in increased accumulation of excretory substances, which may facilitate encystment. In Exp. 4, the ciliates in the CFM group encysted earlier than those in the control and 1/10 CFM groups (Fig. 5). This finding suggests that if they accumulate beyond threshold level, excretory substances from ciliates facilitate encystment. Stolte (1924) suggested that an excreted substance caused encystment in *Blepharisma undulans*. Yonezawa (1986a) also reported an intensive examination of excreted substances as causative agents for encystment in *E. encysticus*. Although increased salt concentration induces encystment in marine species of *Euplotes* (Mélant, 1922; Klee, Garnjobst, 1928, 1937; 1926; Dallai *et al*., 1987), the salt concentration in my experiments remained constant.

At least 2 processes are necessary in causing encystment: suspension of the fission cell cycle, and the beginning of the encystment cycle. I speculate from 3 results (Exp. 1 to 3, Fig. 2 to 4) that if ciliates are kept in conditions that do not support proliferation, encystment is promoted. A deficiency in nutrition necessary for proliferation is the most important inducer of encystment, because I believe that the suspension of the proliferation cell cycle is the first signal for encystment. The results of Exp. 4 (Fig. 5) lead me to think that in the CFM advanced aspects of encystment, such as the morphological changes. Morphologic changes might take place in the oral apparatus of the ciliates, resulting in the inability to eat, as described for *E. encysticus* (Matsusaka *et al*., 1989). Inability to feed has been reported to stimulate *Oxytricha fallax* to encyst (Hashimoto, 1962).

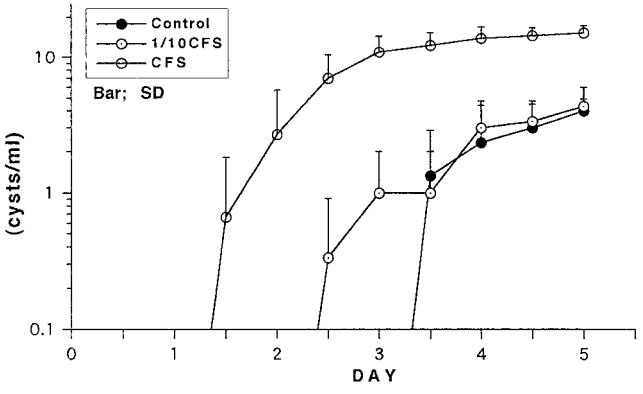

Fig. 5. Effect of cell-free ciliate culture conditioned medium (CFM) on encystment (Exp. 4). The ciliates were transferred to bacteria-free Chalkley's solution, or 1:10 CFM in Chalkley's solution, or CFM. Cysts were counted every 12 hr for 5 days. ●, control group; ○, 1:10 CFM group; ○, CFM group. The data are presented as the mean ± 1 standard deviation (bars) of 3 plates.

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