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Effect of components released from bacteria on encystment in ciliated protozoan *Colpoda* sp.

Chiharu YAMASAKI, Akemi KIDA, Takahiko AKEMATSU and Tatsuomi MATSUOKA*

Institute of Biological Science, Faculty of Science, Kochi University, Kochi 780-8520, Japan

SUMMARY

Ca^{2+}-induced encystment of *Colpoda* sp. was found to be cancelled by bacteria suspended in the surrounding medium. Continuation of the uptake of polystyrene latex particles into the food vacuoles slightly suppressed encystment. When the cells of *Colpoda* containing a large number of food vacuoles filled with Congo-red stained bacteria were transferred into an encystment-inducing medium, they encysted after promptly expelling the contents of the food vacuoles. Supernatant obtained from bacterial suspension, commercial albumin or polypeptone markedly prevented encystment, although free amino acid did not have much of an affect. Boiling or dialysis (MWCO: 10,000) of the supernatant reduced the encystment-suppression effect. These results suggest that the essential factors for the suppression of encystment are components such as peptides or proteins that are released from bacteria suspended in the surrounding medium.

*Corresponding author
Tel: + 81 88 844 8315
Fax: + 81 88 844 8356
E-mail: tmatsuok@cc.kochi-u.ac.jp

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INTRODUCTION

Free-living protozoa must contend with a number of ecological challenges, including lack of food organisms, desiccation, or pollution of the surrounding medium. In order to survive such hostile conditions, some of them are transformed into resting cysts. The processes of encystment and excystment, which involve drastic morphogenetic reconstruction of the cell (Grimes, 1973; Matsusaka, 1979; Walker et al., 1980) that is probably regulated by gene expression or silencing (Matsusaka, 1979; Hirukawa et al., 1998; Suizu and Matsuoka, 1998; Izquierdo et al., 2000), must first be preceded by the detection of environmental signals.

Encystment of soil ciliate, *Colpoda* sp. is induced by an increase in the concentrations of ions contained in the surrounding medium (Watoh et al., 2003); among the ions, Ca^{2+} is the most effective (Yamaoka et al., 2004). Encystment of the cells induced by 1 mM Ca^{2+} is markedly suppressed by bacteria (10^6 - 10^7 cells/ml) suspended in the surrounding medium (Watoh et al., 2003; Yamaoka et al., 2004). However, a much higher concentration of ions invalidates the encystment-suppression effect of bacteria (Yamaoka et al., 2004). The encystment-suppression effect of bacteria is attribut-

able to (1) the simple formation of food vacuoles, (2) the nutrient supply from the food vacuoles, and (3) the suspension of components released from the bacteria in the surrounding medium. The present study examined three elements derived from bacteria that were suspended in the surrounding medium, and revealed that the components released from bacteria were essential for the suppression of encystment.

MATERIALS AND METHODS

Colpoda sp. was isolated from cysts adhering to dried fallen leaves in the field, and it was cultured in an infusion of dried cereal leaves (0.1%) inoculated with bacteria (*Enterobacter aerogenes*) at 23°C in the dark. The bacteria were cultured on agar plates containing 1.5% agar, 0.5% polypeptone, 1% meat extract and 0.5% NaCl. For encystment induction, two- or three-day cultured vegetative cells were rinsed 3 times in each test solution and transferred using a thin glass pipette; 50-60 cells were subsequently suspended in 1.5 ml of each test solution. In order to obtain the substances released from the bacteria, the cultured bacteria were rinsed twice in standard saline solution (1 mM $CaCl_2$, 1 mM KCl, 5 mM Tris-HCl, pH 7.2) by sedimentation (8,000 *g*, 5 min), and subsequently the bacteria were suspended in the solution overnight at room temperature. The supernatant obtained from the bacterial suspension was dialyzed in standard saline solution through two types of membranes (MWCO, 1,000 Da or 10,000 Da; Float-A-Lyzer, Spectrum Laboratories, Inc.) at room temperature for about 48 hr. During dialysis, the surrounding saline solution was replaced by fresh solution several times. The density of bacteria was spectroscopically determined in a diluted bacterial suspension, the value of which had been calibrated by comparing the cell density obtained by counting the colonies on plates with the value of the optical density at 600 nm (OD_{600}). The density of polystyrene latex particles (PLP) (1.1 μm in diameter, Aldrich Chem. Co.) was also spectroscopically determined; its value was calibrated by comparing the density obtained by directly counting the number of particles under a microscope with the value of the optical density at 600 nm (OD_{600}). The rate of encystment was expressed as a percentage of the total number of tested cells (50-60 cells). The columns (points) and attached bars shown in Figs. 2-8 (except for Fig. 4a, open and closed squares) correspond to the means of 4 identical measurements (50-60 cells per measurement) and standard errors, the values of which were obtained 8 hr after encystment induction. Each series of measurements was performed using the cells obtained from each of the batch cultures.

RESULTS AND DISCUSSION

First of all, it was confirmed that *Colpoda* cells actually fed on living bacteria, killed (5-min boiled) bacteria or PLP (Fig. 1). When 3-day cultured *Colpoda* cells containing a few food vacuoles (Fig. 1a) were resuspended in bacteria-free standard saline solution (1 mM $CaCl_2$, 1 mM KCl, 5 mM Tris-HCl, pH 7.2) for 1 hr, the food vacuoles completely disappeared (Fig. 1b). Such starved cells formed a large number of food vacuoles when they were transferred into standard saline solution with suspensions of living bacteria (Fig. 1c), killed bacteria (Fig. 1d), and killed and Congo Red-stained bacteria (Fig. 1e), or PLP (Fig. 1f).

As has been reported previously (Watoh et al., 2003; Yamaoka et al., 2004), the cells of *Colpoda* that were transferred into encystment-induction media quickly encyst within 4-5 hr, and the rate of encysted cells reaches a plateau level. In the present study, therefore, the rate of encysted cells was measured 8 hr after encystment induction.

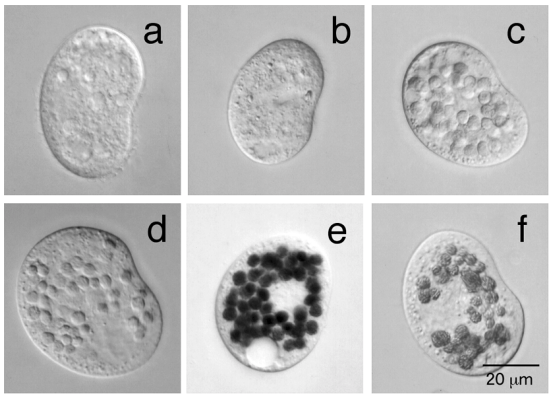

Fig. 1. Photomicrographs showing food vacuoles: (a) three-day cultured *Colpoda* that is partially starved; (b) a cell that was starved for 1 hr in a standard saline solution (1 mM $CaCl_2$, 1 mM KCl, 5 mM Tris-HCl pH 7.2); (c)-(f) cells that were incubated with suspension of bacteria or non-nutrient polystyrene latex particles (PLP, 1.1 μm in diameter). The starved cells (b) were suspended for 1 hr in standard saline solution with suspensions of living bacteria (c), killed (5-min boiled) bacteria (d), killed bacteria subsequently stained with 1% Congo red (e) or PLP (f). The density of the bacteria and PLP were adjusted to 10^7 cells /ml and 3 x 10^6 particles /ml, respectively. In order to take micrographs, cells were paralyzed with 0.05 mM $NiCl_2$ (final concentration) was added to the cell suspension.

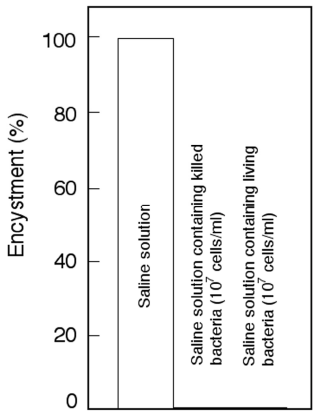

Fig. 2. Suppression of Ca^{2+}-induced encystment of *Colpoda* by the addition of living or killed (5-min boiled) bacteria (*Enterobacter aerogenes*, 10^7 cells/ml each) in the surrounding standard saline solution.

When the *Colpoda* was transferred into standard saline solution, most of the cells encysted (Fig. 2). However, the presence of living or killed (5-min boiled) bacteria (10^7 cells/ml) in the surrounding medium completely cancelled any cyst formation (Fig. 2). As shown in Fig. 3, continuous uptake of PLP into the food vacuoles caused a slight suppression of cyst formation (significantly different from the encystment rate in the standard saline solution; $p < 0.05$, Mann-Whitney test). The result indicates that simple induction of food vacuole formation with PLP is effective for the suppression of encystment, although this effect is not complete, as also suggested in the previous report by Tomaru (2002). The fact that living or killed bacteria completely cancel encystment (Fig. 2) implies that existence of bacteria in the surrounding medium and/or a continuation of the nutrient supply from food vacuoles may be involved in the suppression of encystment. When cells of *Colpoda* containing a large number of food vacuoles that were filled with killed bacteria, which were expected to supply a sufficient nutrient source, were transferred into a fresh standard saline solution (without bacteria), cyst formation was only suppressed slightly (Fig. 4a). Such a slight suppression may be attributable to the simple formation of food vacuoles as induced by PLP. The number of food vacuoles in a vegetative cell quickly decreased after the induction of encystment (Fig. 4a, open squares). In this case, the undigested contents of food vacuoles were expelled just prior to the transformation of the cell into a rounded shape (Fig. 4b, arrows). As shown in Fig. 4a (closed squares), in consequence, very few

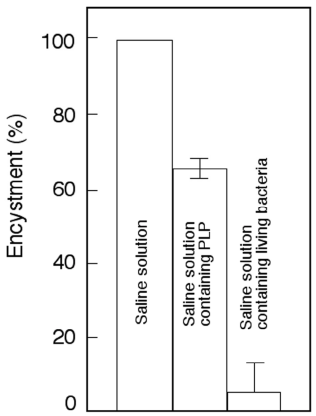

Fig. 3. Suppression of Ca^{2+}-induced encystment of *Colpoda* by the addition of polystyrene latex particles (PLP) in the surrounding standard saline solution. The density of the bacteria and PLP were adjusted to 3×10^6 cells (particles) /ml.

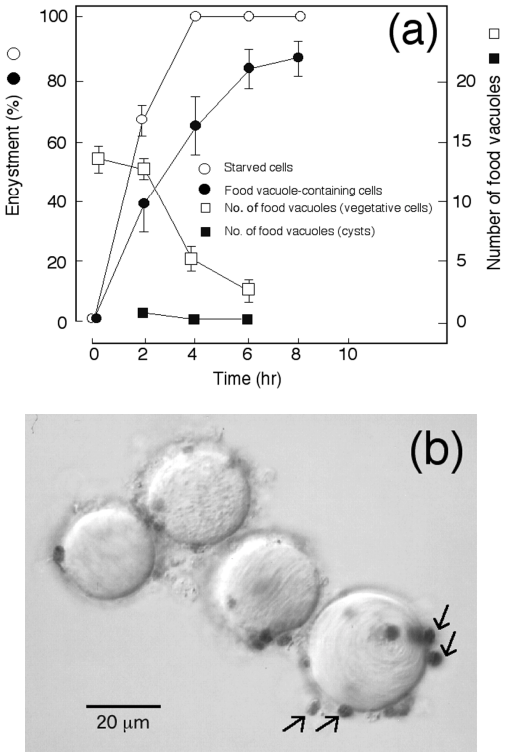

Fig. **4**. Encystment of *Colpoda* cells containing a number of food vacuoles and changes in the number of food vacuoles in a vegetative cell or rounded cell after the induction of encystment with Ca^{2+}. (a) ○, the rate of encystment of starved cells (Ref. Fig. 1b); ●, the rate of encystment of cells containing a number of food vacuoles filled with killed (5-min boiled) and then Congo red (1%)-stained bacteria (Ref. Fig. 1e); □, changes in the number of food vacuoles of vegetative cells; ■, changes in the number of food vacuoles of encysting (rounded) cells. In order to induce food vacuole formation, starved cells were transferred and kept for 1 hr in standard saline solution containing Congo red-stained bacteria (10^7 cells/ml). The cyst formation was induced by suspending the starved cells or food vacuole-bearing cells in the standard saline solution without bacteria. The number of food vacuoles in each cell was counted and expressed as the mean and standard error obtained from 4-10 cells. (b) Photomicrograph of encysting cells that just expelled the contents of food vacuoles. Arrows, expelled Congo Red-stained contents of food vacuoles.

food vacuoles were observed in the rounded cells (early stage of cyst formation). These results suggest that the continuation of the nutrient supply from food vacuoles is not an essential factor for preventing the encystment of *Colpoda*.

Some components released from living or killed (5-min boiled) bacteria prominently cancelled cyst formation (Figs. 5, 6). It is likely that the effective components may be metabolic products released from the living bacteria and/or cellular components excreted by the disrupted cells. When the components obtained from the suspension of living bacteria were dialyzed through a 1,000 Da- or 10,000 Da-cutoff membrane, the encystment-suppressing effect decreased significantly in comparison with that of the crude sample that had not been dialyzed ($p < 0.05$, Mann-Whitney test each), although encystment-suppression activity still remained (Fig. 7). The sample boiled for 5 min also showed significantly reduced encystment-suppression activity in comparison with crude bacterial products ($p < 0.05$, Mann-Whitney test) (Fig.

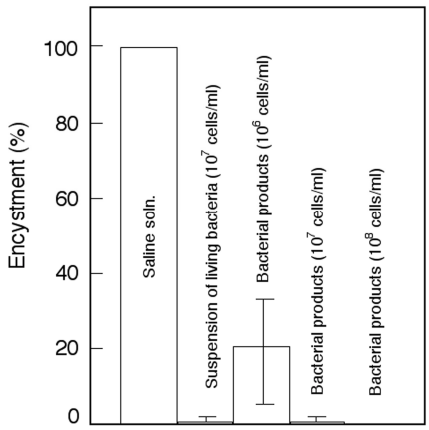

Fig. 5. Suppression of Ca^{2+}-induced encystment of *Colpoda* by the addition of components released from living bacteria. The vegetative cells were transferred into standard saline solutions containing living bacteria (*Enterobacter aerogenes*, 10^7 cells/ml), or containing components released from different densities of bacteria (10^6 - 10^8 cells/ml).

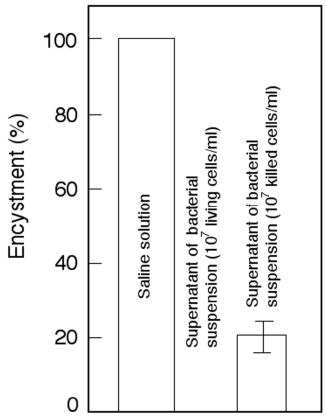

Fig. 6. Suppression of Ca^{2+}-induced encystment of *Colpoda* by the addition of products released from killed (5-min boiled) bacteria. The vegetative cells were transferred into standard saline solutions containing living or killed (boiled for 5 min) bacteria (*Enterobacter aerogenes*, 10^7 cells/ml).

7). These results suggest that not only small molecules but also large molecules such as proteins are involved in the suppression of encystment.

Commercial albumin (from chicken egg) and polypepton remarkably suppressed encystment of the cells at concentrations above 1 mg/ml (Fig. 8). On the other hand, glycine only slightly suppressed encystment at concentrations of less than10 mM (0.75 mg/ml) (Fig. 8). These results suggest that the encystment-suppression effect induced by proteins or peptides may be responsible for some side-chain groups of amino acid residues or a specific conformation dependent upon a short common sequence of amino acids. It is likely that this encystment-suppression effect may not be responsible for the common structure of amino acids such as amino and carboxyl groups. In the measurements shown in Fig. 8, the cells were transferred into the solutions containing albumin or polypepton every 1.5 hr, because contaminated bacteria may proliferate in these solutions; when the bacteria were inoculated into these solutions, the number of bacteria increased twice per 4 hr in albumin solution (10 mg/ml), and twice per 1.2 hr in polypepton solution (10 mg/ml) at room temperature. On the other hand, the inoculated bacteria did not grow in the supernatant obtained from bacterial suspension (10^7 cells/ml). The mean of density of bacteria contaminated in the test solutions with suspension of *Colpoda* (50-60 cells/ml) was 13 cells/ml (n=3), the value of which was obtained by counting the colonies on culture plates. On the other hand, the bacterial density less than 10^3 cells/ml did not suppress Ca^{2+}-induced encystment of *Colpoda*. In this case, mean values of the encystment rate (n=4) were above 78.5% when the *Colpoda* cells were transferred and kept for 8 hr in the standard saline solutions containing less than10^3 bacteria/ml (61.9% at 10^4 bacteria/ml; 78.5% at 10^3 bacteria/ml; 87.9% at 10^2 bacteria/ml; 89.3% in case of no

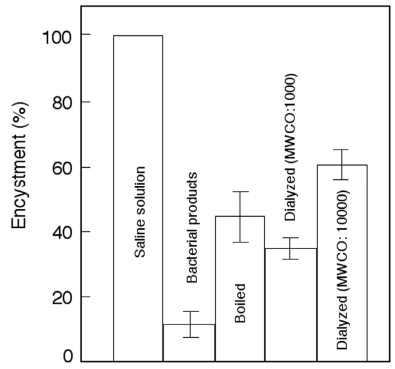

Fig. 7. Effects of boiling or dialysis of products obtained from living bacteria on the Ca^{2+}-induced encystment of *Colpoda*. "Saline solution," standard saline solution; "Bacterial products," supernatant obtained from suspension (standard saline solution) of living bacteria (*Enterobacter aerogenes*, 10^7 cells/ml); "Boiled," supernatant boiled for 5 min; "Dialyzed (MWCO:1000)," supernatant cutoff below 1,000 Da; "Dialyzed (MWCO:10000)," supernatant cutoff below 10,000 Da. In order to obtain bacterial components in the supernatant, the living bacteria (10^7 cells/ml) were suspended overnight in the standard saline solution at room temperature.

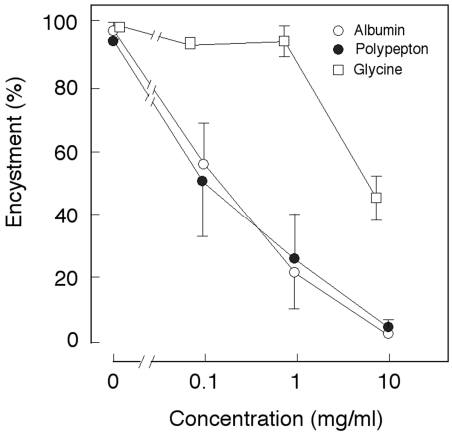

Fig. 8. Effects of albumin from chicken egg (○), polypepton (●), or glycine (□) on the Ca^{2+}-induced encystment of *Colpoda*. These chemicals were dissolved in standard saline solution. In order to eliminate the proliferation of bacteria in the test solutions (standard saline solution) containing polypepton or albumin, the cells were transferred into fresh solution every 1.5 hr. The solution containing polypepton had been sterilized.

addition of bacteria); there is no significant difference among the encystment rates at the bacterial density less than 10^3 cells/ml ($p > 0.05$, Kruskal-Wallis test). In consequence, a small number of bacteria (10-20 cells/ml) in the test solution may not affect the rate of encystment shown in Fig. 8.

In conclusion, one essential factor in preventing Ca^{2+}-induced encystment of *Colpoda* is the presence of molecules such as proteins, peptides or other small molecules released from living or disrupted bacteria. In addition, the simple formation of food vacuoles is apt to suppress encystment, although it is not important whether the contents of the vacuoles are nutrient or non-nutrient particles.

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