J. Protozool. Res. 15, 7-13(2005)

Studies on the resting cyst of ciliated protozoan *Colpoda cucullus*: resistance to temperature and additional inducing factors for en-or excystment

Hajime Maeda, Takahiko Akematsu, Risa Fukui and Tatsuomi Matsuoka*

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

*Corresponding author: Tatsuomi Matsuoka, E-mail tmatsuok@cc.kochi-u.ac.jp

Key words: *Colpoda*; encystment; excystment; resistance to temperature; Ca^{2+}; porphyrins

ABSTRACT

The resting cysts of *Colpoda cucullus* were resistant to not only drying, but high and low temperatures. The critical temperatures for the survival of wet cysts ranged from 40 °C (3-hr exposure) to 45 °C (10-min exposure), and those of the dried cysts ranged from 80 °C (3-hr exposure) to 100 °C (30-min exposure). Frozen (-30 °C, 3 hr) and remelted wet cysts also survived. Ca^{2+}-induced resting cysts excysted when the external Ca^{2+} was removed. However, the excystment-inducing effect by Ca^{2+} removal was canceled by the addition of cations such as Na^{+}, K^{+}, and Mg^{2+} in the surrounding medium. When the mature cysts transformed in a diluted buffer without any other salts were temporally exposed to Ca^{2+} and subsequently resuspended in the buffer without Ca^{2+}, excystment was induced in a majority of cysts. The addition of chlorophyll-derived molecules in the surrounding medium induced excystment, but suppressed encystment. This suggests that the excystment-inducing and encystment-suppressing activities of cereal infusion are attributed to water-soluble porphyrins derived from chlorophyll. The encystment was induced when the vegetative cells were suspended at a high density, and the substitution of non-living particles such as polystyrene latex particles (PLP) for the living cells also showed an encystment-inducing effect. The result suggests that mechanical cell-to-cell contact induces encystment.

INTRODUCTION

The cysts of terrestrial *Colpoda* are necessarily resistant to drying. In addition, the cysts of some species of *Colpoda* are resistant to high or low temperatures (Taylor and Strickland 1936). In the present study, the resistance of the resting cysts of *Colpoda cucullus* to high or low temperatures was examined to reconfirm the previous studies.

The encystment of *Colpoda* sp. which is identical with the clone (*C. cucullus*) employed in the present study, is induced by an increase in external cations such as Ca^{2+} (Watoh *et al*. 2003, Yamaoka *et al*. 2004), which is suppressed by the addition of components released from bacteria (Watoh *et al*. 2003, Yamasaki *et al*. 2004), components contained in cereal infusion, or artificial porphyrin analogues such as chlorophyllin-Cu (Tsutsumi *et al*. 2004). On the other hand, the excystment of *C. cucullus* is elicited by the addition of hay infusion (Haagen-Smit and Thimann 1938), cereal infusion (Watoh *et al*. 2003; Tsutsumi *et al*. 2004) or chlorophyllin-Cu (Tsutsumi *et al*. 2004). These imply that the effective components contained in the cereal infusion are water-soluble porphyrins which may be mainly derived from chlorophyll. In the present study, therefore, the excystment-inducing and encystment-suppressing effects of water-soluble components derived

from purified chlorophyll were examined.

In *Colpoda*, one of the encystment-inducing factors is an increase in the external Ca^{2+} concentration (Watoh et al. 2003; Yamaoka et al. 2004) which may be a signal foretelling the forthcoming desiccation. In contrast, the removal of Ca^{2+} is expected to induce excystment. In the present study, the excystment-inducing effect of the removal of Ca^{2+} was examined.

Overpopulation is known as one of the encystment-inducing factors (Strickland 1940; Corliss and Esser 1974). We confirmed that in *C. cucullus*, the encystment was also induced by overpopulation. The present study aimed to reveal what kinds of elements produced by overpopulation (for example, a simple mechanical contact, the cell-to-cell chemical interaction, or the accumulation of encystment-inducing substances excreted by cells) are effective for encystment induction.

MATERIALS AND METHODS

Cell culture and en- or excystment induction

Colpoda cucullus was cultured in an infusion of *dried* cereal leaves (0.1 %) inoculated with bacteria (*Enterobacter aerogenes*) at 23 ^{o}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. The resting cysts employed in Fig. 1 were obtained by suspending 3-day-cultured vegetative cells in a saline solution containing 1 mM $CaCl_2$, 1 mM KCl and 5 mM Tris-HCl (pH 7.2) and kept for more than 1 month. In other experiments on excystment (Figs. 2-4), in order to obtain the resting cysts, the 3-day-cultured vegetative cells were suspended either in a 0.1 mM Tris-HCl buffer (pH 7.2) containing 1 mM $CaCl_2$ at the cell density less than 100 cells/ml (Figs. 2, 4), or a 0.1 mM Tris-HCl (pH 7.2) buffer without any other salts at the cell density of more than 3,000 cells/ml (Fig. 3), and then kept for a month. In order to examine the encystment (excystment) rates (%), the vegetative cells (cysts) were rinsed twice in media containing tested components, and 50-100 cells (cysts) were then suspended in 1 ml of each test solution using a thin glass pipette. The rates of encystment (excystment) were examined at 10 hr after the onset of encystment (excystment) induction, and expressed as a percentage of the total number of tested cells (50-100 cells). Columns (points) and bars correspond to the means of five (Figs. 1a, b) or four (Figs. 2-5) identical hmeasurements (50-100 cells per measurement) and standard errors, respectively.

Purification of chlorophyll

Chlorophyll was extracted with acetone from the homogenate of spinach leaves. The extracts were concentrated with a rotary evaporator (Rotavapor, Sibata), subsequently applied on a TLC plate (Whatmann, 60A LK6F), and developed with a mixture of petroleum ether and acetone (4:1, vol/vol). Both of the spots corresponding to chlorophyll a and chlorophyll b were scraped together and then suspended in acetone. The supernatant obtained by centrifugation (8,000 g, 5 min) of the suspension was decanted to dry using a rotary evaporator. The dried sample (2.1 mg) was dissolved in 1 ml of ethanol, and 50 ml of water was added. The mixture was then boiled for 10 min to evaporate the ethanol. The chlorophyll suspension was autoclaved several times, partially disintegrating the chlorophyll molecules so they became soluble in water. Concentrated Tris-HCl was added into the sample, producing 0.1 mM Tris-HCl (pH 7.2) containing chlorophyll (42 μg/ml). The sample was filtrated because insoluble components still remained.

RESULTS AND DISCUSSION

Resistance of resting cysts to high or low temperatures

When the resting cysts suspended in the saline solution containing 1 mM $CaCl_2$, 1 mM KCl and 5 mM Tris-HCl (pH 7.2) were exposed to 40 °C for 3 hr, most of them survived; that is, most of them excysted within 10 hr when they were transferred into the 0.1% cereal infusion. However, when the cysts were exposed to 43 °C for 3 hr, no cell excysted , indicating the cysts were killed (Fig. 1a). In the case of 10-min warming, the critical temperature for survival was 45 °C (Fig. 1a). Some of the dried cysts withstood temperatures above 80 °C (Fig. 1b). The cysts showed resistance to lower temperatures (Figs. 1a, b). Most of the cysts withstood the lower temperatures, even when the cysts were kept for 3 hr at –30 °C (Fig. 1a). These results are basically consistent with previous observations (Taylor and Strickland 1936), although the dried cysts did not withstand temperatures up to 120 °C, as reported by Taylor and Strickland (1936).

The temperatures of the air in the shade out of the sun, water (1-cm depth) filled in a container (30 cm x 20 cm x 12-cm height) placed on a rooftop, and a portion of the soil surface that lay under fallen leaves upon which the sun was shining directly were measured on August 13, 2004 (fine weather) (Fig. 1c). The maximum temperatures were 32 °C, 37 °C and 41 °C in the air, water, and soil surface, respectively.

The upper limit of temperature in which the wet cysts could survive was 40 °C (in the case of 3-hr

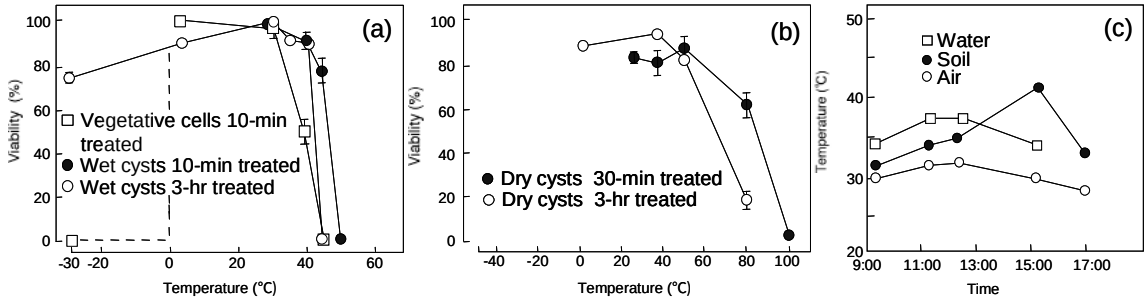

Fig. 1. The viability of the resting cysts against high and low temperatures (Figs. 1a, b) and temperature changes on August 13, 2004 in Kochi prefecture, Japan (Fig. 1c). (a), the viability of the vegetative cells (open squares) and the cysts (open and closed circles) susp ended in a saline solution containing 1 mM $CaCl_2$, 1 mM KCl and 5 mM Tris-HCl (pH 7.2) (wet cysts). (b), viability of dried cysts. The viability of the cysts was expressed as the excystment rage (%) in 10 hr after the onset of induction. In order to induce excystment, 50-100 cysts (1-month old or more) were transferred into 1 ml of 0.1% fresh cereal infusion filled in watch glasses, after they were exposed to various temperatures.

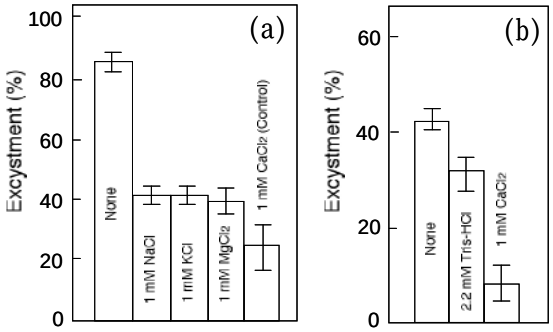

Fig. 2. Excystment-suppressing effect by external ions. The cysts were transferred into various solutions containing 0.1 mM Tris-HCl (pH 7.2), except for a column labeled with '2.2 mM Tris-HCl' in Fig. 2 (b). "None" labeled in the columns indicates 0.1 mM Tris-HCl (pH 7.2) without any other salts. The employed resting cysts were obtained by suspending the vegetative cells in a 0.1 mM Tris-HCl buffer (pH 7.2) containing 1 mM $CaCl_2$ at a cell density less than 100 cells/ml.

exposure) (Fig. 1a) and that for dried cysts was 80-100 ^{o}C (Fig. 1b). Such critical temperatures seem to be enough for the resting cysts to survive, because the temperature of the pools may not reach 40 ^{o}C in summer, and the surface of the soil does not reach 80 ^{o}C (Fig. 1c). The fact that the viability of the cysts exposed to high temperatures for 3 hr is lower than that of cysts exposed for a short time (Fig. 1) indicates that the insulation by the cyst wall is not perfect. However, the cysts kept for 3 hr at -30 ^{o}C survived (Fig. 1a). Presumably, the highly condensed cytoplasm of the resting cysts prevent the production of large crystals of ice. The resistance of the cysts to freezing and thawing is quite important, because pools and soil surfaces are often frozen in the wintertime in Japan.

Effects of salts (ions) on excystment

The resting cysts which had been encysted in a 0.1 mM Tris-HCl buffer containing 1 mM $CaCl_2$ and kept for a month were transferred into the various saline solutions containing 0.1 mM Tris-HCl (pH 7.2) (Fig. 2). The simple removal of $CaCl_2$ from the encystment-inducing medium elicited a prominent excystment (Fig. 2a, "None"); in this case, the rate was significantly different from the "Control" (the composition identical with the encystment-inducing medium) ($p < 0.05$, Mann-Whitney test). On the other hand, the excystment induced by the removal of $CaCl_2$ was significantly suppressed ($p < 0.05$, Mann-Whitney test) by the presence of other salts such as NaCl, KCl and $MgCl_2$ (Fig. 2a). Some of the cysts excysted even when they were transferred into the solution identical with encystment-inducing medium (Fig. 2a). In this case, the excystment is probably elicited by mechanical stimulation (pipetting).

Tris-HCl buffer at 2.2 mM (pH 7.2) that contains 2 mM Cl^- (identical molar concentration with Cl^- produced by the ionization of 1 mM $CaCl_2$) did not suppress excystment (Fig. 2b); there was no significant difference between "None" and "Tris-HCl" ($p > 0.05$, Mann-Whitney test). The encystment-suppressing effect by the salts in the surrounding medium can possibly be attributed to osmolality. The tris-HCl buffer at 2.2 mM whose osmolality was greater than that of the 1 mM $CaCl_2$ solution did not significantly suppress excystment (Fig. 2b). The results suggest that the excystment-suppressing effect by salts is mainly attributed to cations such as Ca^{2+}, Na^+, K^+ and Mg^{2+}, and not to osmolality in low ionic concentrations.

In order to reveal whether or not the temporal exposure of the mature cysts to salts and the removal of them induce excystment, the 30-day aged cysts which had been encysted by overpopulation (more than 3,000 cells/ml) in a 0.1 mM Tris-HCl buffer (pH 7.2) were transferred into 1 mM saline solutions containing 0.1 mM Tris-HCl buffer, kept for 10 days, and finally transferred into 0.1 mM Tris-HCl buffer (pH 7.2) without any other salts. As shown in Fig. 3, only the temporal exposure of the cysts to the $CaCl_2$ solution had a prominent excystment-inducing effect, and $MgCl_2$ showed a slight effect; each significantly different from the 'Control' (Mann-Whitney test, $p < 0.05$). These results imply that Ca^{2+}-binding receptor sites may occur on the resting mature cyst, which may be activated by two steps of binding and the dissociation of Ca^{2+} (Fig. 6a).

Effects of chlorophyll-derived molecules on excystment and encystment

10^{-4} M chlorophyllin-Cu (chlorophyllin coppered, sodium salts) prominently induces encystment and suppresses the Ca^{2+}-induced encystment of *Colpoda* (Tsutsumi et al. 2004). The 0.1 mM Tris-HCl buffer containing chlorophyll (42 μg/ml) produces 4.7×10^{-5} M chlorophyll solution if it is completely dissolved. Chlorophyll-derived water-soluble components prominently induced encystment (approximately 80%) (Fig.

4a) and suppressed Ca^{2+}-induced encystment (Fig. 4b); each significantly different from the "Control" ($p < 0.05$, Mann-Whitney test). Judging from the fact that a kind of porphyrin, chlorophyllin-Cu, is an effective molecule for inducing excystment and for suppressing encystment, the effective components derived from chlorophyll are probably water-soluble porphyrins, and the excystment-inducing or encystment-suppressing activity of dried leaves may be attributed to chlorophyll-derived porphyrins.

Effects of overpopulation on encystment

When the vegetative cells were suspended in 0.1 mM Tris-HCl buffer (pH 7.2) at a high density, a large number of cells encysted (Fig. 5a); significantly different from one another; $p < 0.05$, Kruskal-Wallis test). The cell-free medium obtained from the cell suspensions (3,000 cells/ml, 30,000 cells/ml) did not have encystment-inducing activity (Fig. 5b); there was no significant difference between "None" and "Cell-free

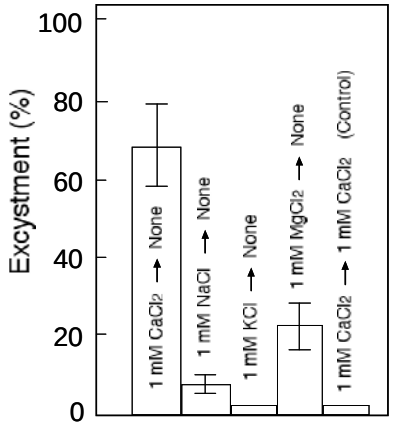

Fig. 3. Excystment-inducing effect of temporal addition and subsequent removal of external salts (ions). The 30-day aged mature cysts which had been encysted in a 0.1 mM Tris-HCl buffer (pH 7.2) without any other salts were transferred into the 0.1 mM Tris-HCl buffer (pH 7.2) containing salts (ions), subsequently kept for 10 days, and finally resuspended in 0.1 mM Tris-HCl buffer (pH 7.2) without any other salts.

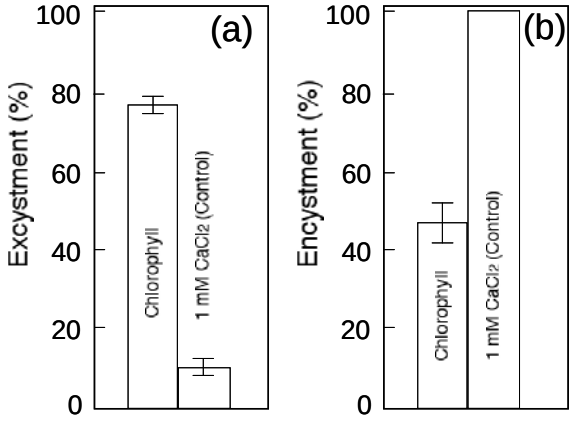

Fig. 4. Excystment-inducing (a) and encystment-suppressing (b) effects of chlorophyll-derived components (42 µg/ml; 4.7×10^{-5} M) which were dissolved in 0.1 mM Tris-HCl buffer (pH 7.2) containing 1 mM $CaCl_2$. (a): The employed resting cysts were obtained by suspending the vegetative cells in a 0.1 mM Tris-HCl buffer (pH 7.2) containing 1 mM $CaCl_2$ at a cell density less than 100 cells/ml (b): The cells were transferred into fresh solutions every hour to eliminate the effect of the proliferation of bacteria, because encystment is suppressed by bacterial components (Yamasaki et al. 2004).

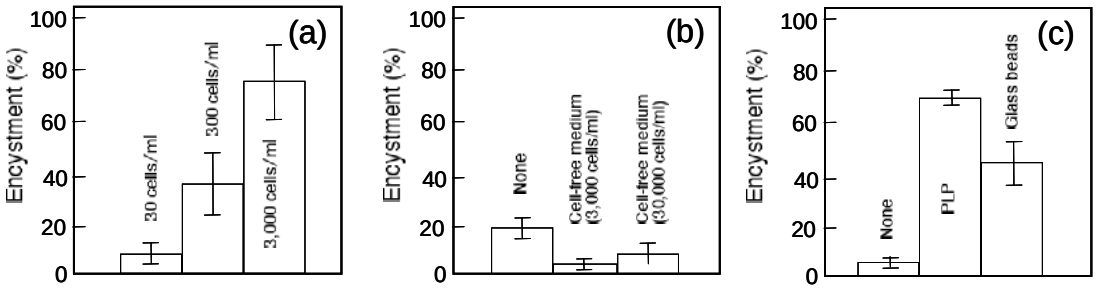

Fig. 5. Encystment-inducing effects of overpopulation of vegetative cells (a), cell-free media obtained from suspensions of the vegetative cells kept at a high density (3,000 cells or 30,000 cells/ml) (b), and the substitution of polystyrene latex particles (PLP) and glass beads for living vegetative cells (c). (b), (c): The density of the tested cells was adjusted to 50-100 cells/ml. The PLP and glass beads were suspended at the density of 3000 particles/ml.

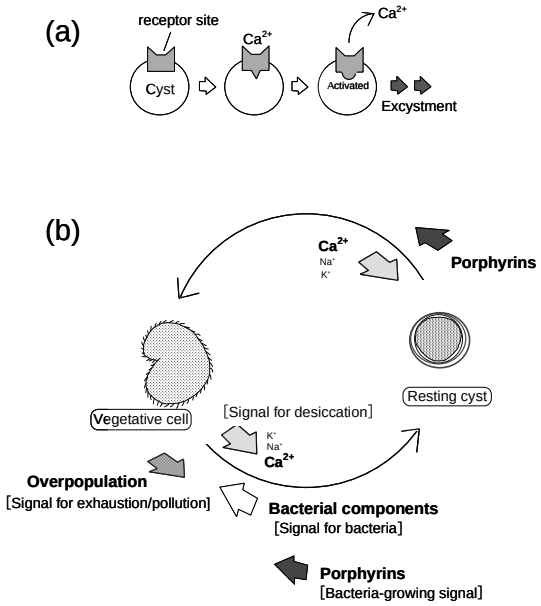

Fig. 6. (a): A presumed receptor site of mature cysts for detecting the addition and removal of external Ca^{2+}. (b): A schematic diagram drawn based on the present study and our recent results, showing the environmental elements controlling the encystment and excystment of *C. cucullus*.

media" ($p > 0.05$, Mann-Whitney test). The suspension (3,000 particles/ml) of polystyrene latex particles (PLP) (26 μm in diameter, Aldrich Chem. Co.) or glass beads (45 μm in diameter, Wako) markedly induced encystment (Fig. 5c); each significantly different from "None" ($p < 0.05$, Mann-Whitney test). These results suggest that encystment induction by overpopulation might be responsible for mechanical stimulation due to cell-to-cell contact.

In Fig. 6 (b), the environmental factors controlling the encystment and excystment cycle of C. cucullus are summarized, based on the present study and our recent results. The increase in the concentration of cations such as Ca^{2+} (Yamaoka et al. 2004), which is one of the encystment-inducing factors, may be the signal which foretells forthcoming desiccation. Overpopulation may be the signal for the forthcoming accumulation of metabolic wastes and the exhaustion of foods. However, bacteria-derived components excreted in the surrounding medium, which is one of the encystment-suppressing factors, are the signal indicating the existence of bacteria in the surrounding medium (Yamasaki et al. 2004). In addition, certain kinds of porphyrins indicate an environment adequate for the growth of bacteria.

REFERENCES

Corliss, J. O. & Esser, S. C. 1974. Comments on the role of the cyst in the life cycle and survival of free-living protozoa. Trans. Amer. Micros. Soc. 93: 578-593.

Haagen-Smit, A. J. & Thimann, K. V. 1938. The excystment of *Colpoda cucullus*. I. The chemical nature of the excysting factors in hay infusion. J. Cell. Comp. Physiol. 11: 389-407.

Strickland, A. G. R. 1940. The effect of concentration of *Colpoda duodenaria* on the time required for encystment in food-free medium. Physiol. Zool. 13: 356-365.

Taylor, C. V. & Strickland, A. G. R. 1936. Effects of high vacua and extreme temperatures on the cysts of *Colpoda* cucullus. Physiol. Zool. 9: 15-26.

Tsutsumi, S., Watoh, T., Kumamoto, K., Kotsuki, H. & Matsuoka, T. 2004. Effects of porphyrins on encystment and excystment in ciliated protozoan *Colpoda* sp. Jpn. J. protozool. 37:119-126.

Watoh, T., Yamaoka, M., Nagao, M., Oginuma, K. & Matsuoka, T. 2003. Inducing factors for encystment and excystment in ciliated protozoan *Colpoda* sp. Jpn. J. Protozool. 36:105-111. (in Japanese)

Yamaoka, M., Watoh, T. & Matsuoka, T. 2004. Effects of salts concentration and bacteria on encystment induction in ciliated protozoan *Colpoda* sp. Acta Protozool. 43:93-98.

Yamasaki, C., Kida, A., Akematsu, T. & Matsuoka, T. 2004. Effect of components released from bacteria on encystment in ciliated protozoan *Colpoda* sp. Jpn. J. Protozool. 37: 111-117.