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Encystment-inducing factor “starvation” in ciliated protozoan *Colpoda cucullus*

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Summary

“Starvation” is known to be a common factor in encystment induction among a number of protozoans. Encystment induction by starvation may be responsible for consumption of encystment-suppressing nutrient molecules and/or their metabolic products. Encystment of *Colpoda cucullus* was suppressed and cells temporarily proliferated as a result of addition of D-glucose in the surrounding medium, whereas such encystment suppression and cell proliferation were cancelled in the presence of phlorizin, an inhibitor of D-glucose uptake. The results suggest that D-glucose or its metabolic products may affect the intracellular signaling pathway responsible for the encystment induction. When ATP synthesis was blocked by CCCP, oligomycin, or NaN_3, the encystment suppression by D-glucose tended to be canceled. It is possible that final metabolic products of D-glucose including ATP, might be related to the suppression of encystment induction.

Key words: *Colpoda*, encystment suppression, D-glucose, phlorizin, nutrients, starvation

Introduction

The resting cyst formation (encystment) of terrestrial ciliates *Colpoda* is induced by an increase in mainly external Ca^{2+} concentration (Yamaoka et al., 2004) or overpopulation of vegetative cells (Barker and Taylor, 1931; Strickland, 1940; Maeda et al., 2005). It is known that the Ca^{2+}-induced encystment of *Colpoda* is suppressed when bacteria exist in the surrounding medium (Barker and Taylor, 1931). In this case, the suppression of encystment is mainly responsible for the release of some molecules such as peptides released from bacteria (Yamasaki et al., 2004; Kida et al., 2009). In addition, the components contained in plant leaves, such as porphyrins including chlorophyll-derived molecules (Tsutsumi et al., 2004; Maeda et al., 2005) also suppress encystment induction. Presumably, such encystment-suppressing elements contained in the surrounding medium may suppress encystment by affecting either certain receptor molecules on the plasma membrane, or intracellular signaling pathways leading to the induction of encystment after they are internalized.

When *Colpoda* vegetative cells are suspended in the medium (e.g., pure water or 1 mM Tris-HCl

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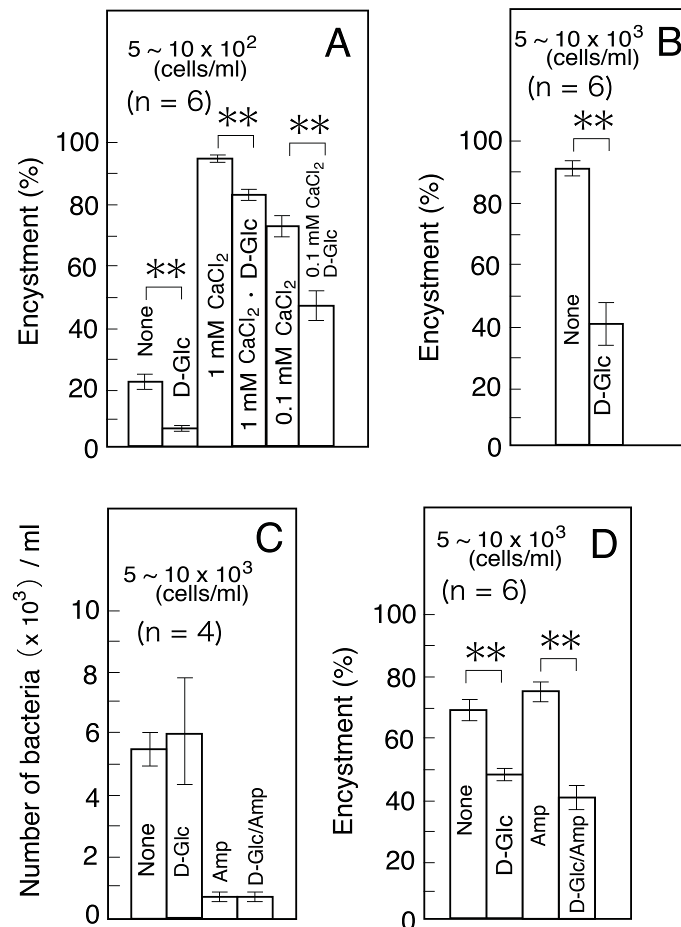

Fig. 1. Suppression of encystment of *C. cucullus* by D-glucose. (A) Spontaneous (the leftmost set of columns) and Ca^{2+} -induced encystment at the low cell density (500~1,000 cells/ml) and suppression by addition of 0. 5 mM D-glucose (D-Glc). (B) Overpopulation (5,000~10,000 cells/ml)-induced encystment and its suppression by addition of 0. 5 mM D-glucose. (C) Suppression of proliferation of bacteria contained in *Colpoda*-containing (5,000~10,000 cells/ml) test solutions by addition of 50 μg/ml (final concentration) ampicillin (Amp). Columns indicate the density of bacteria contained in the test solutions 8hr after onset of encystment induction. (D) Effect of elimination of bacteria contained in the test solutions by addition of 50 μg/ml (final concentration) ampicillin (Amp) on D-glucose-induced encystment suppression of *Colpoda* (right two set of columns). Left two sets of column (control): Overpopulation (5,000~10,000 cells/ml)-induced encystment and its suppression by addition of 0. 5 mM D-glucose.

[pH 7.2]) to which neither an encystment-inducing nor encystment-suppressing element is added, encystment occurs slowly (unpublished data). In the present paper, such cyst formation is termed 'spontaneous encystment' that is responsible for 'starvation,' which has been believed to be a common encystment-inducing factor among many protozoans (Corliss and Esser, 1974). Therefore, 'encystment induction by starvation' seems to be synonymous with a cancellation of encystment suppression caused by a consumption or removal of encystment-suppressing nutrient molecules. However in this case, micromolar Ca^{2+} ions contaminating the surrounding medium may be responsible for encystment induction. The present study focuses on suppression of encystment induction by an energy-source nutrient D-glucose, and discusses the mechanism of this suppression.

Material and methods

Organisms

Vegetative cells of *Colpoda cucullus* were cultured in 0.05% (w/v) cereal infusion inoculated

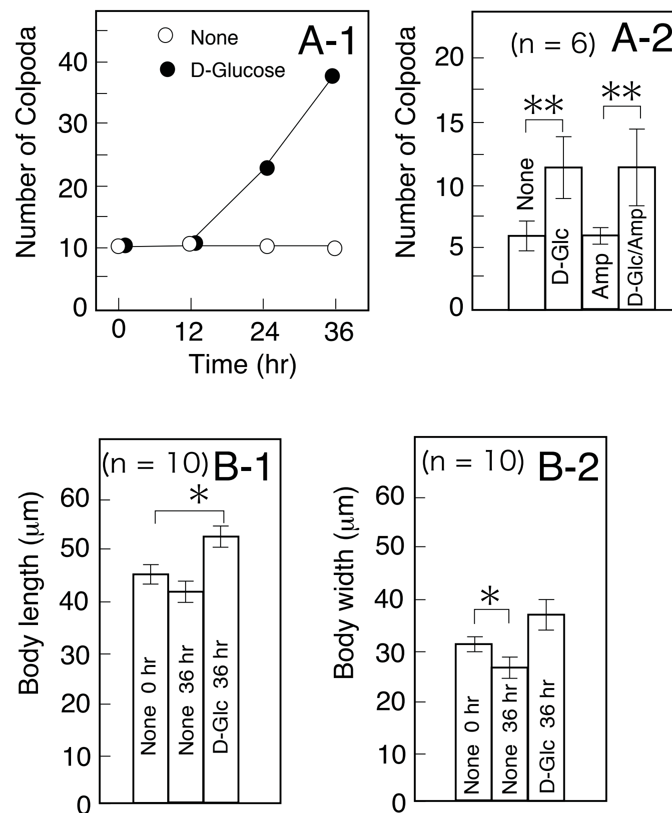

Fig. 2. Growth of vegetative cells of *C. cucullus* in the presence of D-glucose. (A-1) Growth curve of vegetative cells suspended in 1 mM Tris-HCl buffer (pH 7.2) containing 0.5 mM D-glucose (●) or without glucose (○). Ten cells of *Colpoda* were inoculated in 100 µl of each medium. (A-2) Effect of elimination of bacteria contained 7 in test solution by the addition of 50 µg/ml (final concentration) ampicillin (Amp) on growth of *Colpoda*. The number of cells were counted in 36 hr after the 10 cells were inoculated in a 100 µl fresh culture medium. (B) Body length (B-1) and body width (B-2) of vegetative cells cultured in 1 mM Tris-HCl buffer (pH 7.2) without D-glucose for 0 hr ('None 0 hr') or 36 hr ('None 36 hr'), or cultured in 1 mM Tris-HCl (pH 7.2) containing 0.5 mM D-glucose for 36 hr ('D-Glc 36 hr').

with bacteria (*Enterobacter aerogenes* or *Klebsiella pneumoniae*). The bacteria were cultured on agar plates containing 1.5% agar, 0.5% polypepton, 1% meat extract and 0.5% NaCl. For encystment induction, vegetative cells cultured for 1-2 days were collected by centrifugation (1,500 g, 2 min), washed 2-3 times in 1 mM Tris-HCl (pH 7.2), and then transferred into 200 µl volumes of test solutions at the cell density of 500~1,000 cells/ml or 5,000~10,000 cells/ml (overpopulation).

The density of bacteria (>10^6 cells/ml) in the culture medium was spectroscopically determined. The value of this density 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}). When the bacterial density was lower than 10^6 cells/ml, the number of colonies on the culture plates was directly counted.

Chemicals

Phlorizin (Wako Pure Chemical Industries), carbonyl cyanide 3-chlorophenylhydrazone (CCCP, Nacalai) and oligomycin (Wako Pure Chemical Industries) were dissolved in dimethyl sulfoxide (DMSO) to give 100 mM, 100 µM and 10 µg/ml stock solutions, respectively, and 10 µl of each stock solution was added to 10 ml volumes of test solutions to produce final concentrations of test solutions of 100 µM, 0.1 µM and 0.01 µg/ml, respectively (final concentration of DMSO at 0.1%). Sodium azide and ampicillin (Wako Pure Chemical Industries)

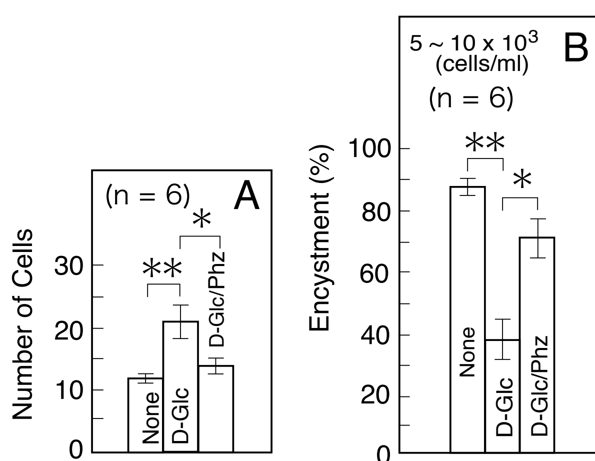

Fig. 3. Effects of phlorizin on cell proliferation (A) and suppression of encystment (B) in *C. cucullus* induced by addition of D-glucose. (A) Number of cells cultured for 36 hr in 1 mM Tris-HCl buffer (pH 7.2) without any other components ('None'), containing 0.5 mM D-glucose ('D-Glc'), or containing 0. 5mM D-glucose and 0.1 mM phlorizin ('D-Glc/ Phz). Ten *Colpoda* cells were inoculated in 100 μl of each test solution at the onset of the cultures. (B) Cancellation of glucose-mediated encystment-suppressing effect by phlorizin. The cells were suspended at a high density (5,000~10,000 cells/ml) in 1 mM Tris-HCl buffer (pH 7.2) without any other components ('None'), containing 0.5 mM D-glucose ('D-Glc'), or containing 0. 5mM D-glucose and 0.1 mM phlorizin ('D-Glc/ Phz). The rates of encystment (%) were measured 8 hr after the onset of induction.

were dissolved in pure water to produce 0.1 M and 20 mg/ml stock solutions, respectively.

Encystment assays

From 80 to120 cells were randomly chosen, the number of encysting cells were counted, and the rate of encysting cells was expressed as a percentage of the total number of cells. Points (columns) and attached bars in Figs 1, 2A-2, 2B and 3 correspond to the means and SE of 4-6 identical measurements (Figs 1, 3) or 10 cells (Fig. 2B), respectively. In Fig. 4, the columns and attached bars correspond to the means and SE, respectively, of 6 identical measurements (A-1~C-1) or 6 determinations of encystment-suppression rates (A-2~C2) obtained from the data in the left figures (A-1~C-1). In Figs 1-4, all test solutions contained at least 1 mM Tris-HCl (pH 7.2), and some test solutions (Figs 3, 4A, 4B) contained 0.1% DMSO. Other components of the test solutions are indicated in figures. The rates of encystment (%) were measured 8 hr after the onset of induction. Asterisks (*) and double asterisks (**) represent significant differences among columns at $p < 0.05$ and $p < 0.01$ (Mann-Whitney test).

Results and discussion

Spontaneous encystment (Fig. 1A, leftmost two columns), Ca^{2+}-mediated (Fig. 1A, middle two columns and rightmost columns) and over-population-mediated (Fig. 1B) encystment were significantly suppressed in the presence of D-glucose (D-Glc), although encystment induced by higher concentrations of Ca^{2+} (1 mM) was not as markedly suppressed (Fig. 1A, middle two columns), as has been previously reported (Kida et al., 2009). It is known that bacteria suspended in the surrounding medium at a high density suppress the encystment induction, but bacteria at a lower density (< 10^3 cells/ml) do not affect encystment induction (Yamasaki et al., 2004). Therefore, the suppression of encystment induction by D-glucose is possibly due to the bacterial proliferation caused by a supply of D-glucose. With the addition of 50 μg/ml ampicillin (final concentration), the bacterial density was reduced below 10^3 cells/ml (Fig. 1C). In such conditions, encystment was significantly suppressed in the presence of D-glucose (Fig. 1D). This result suggests that the suppression of encystment in the presence of D-glucose cannot be

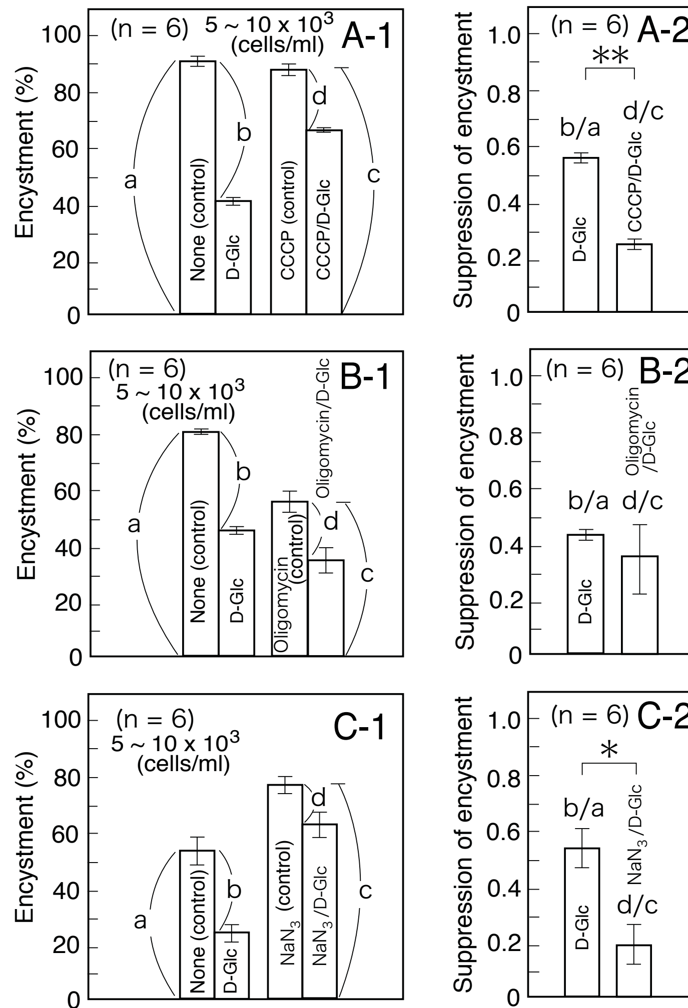

Fig. 4. Effects of the inhibition of ATP synthesis on D-glucose-mediated suppression of encystment of *C. cucullus.* (A) 0.1 μM CCCP ; (B) 10 μg/ml oligomycin; (C) 10 μM sodium azide (NaN3). Each left set columns in (A-1), (B-1) and (C-1) shows overpopulation (5,000~10,000 cells/ml)-induced encystment ('None') in 1 mM Tris-HCl buffer (pH 7.2) and its suppression by addition of 0.5 mM D-glucose ('D-Glc). Each right set of columns shows overpopulation (5,000~10,000 cells/ml)-induced encystment in 1 mM Tris-HCl buffer (pH 7.2) containing ATP synthesis inhibitors ('CCCP', 'Oligomycin', 'NaN3') and its suppression by addition of 0.5 mM D-glucose ('CCCP/D-Glc', 'Oligomycin/D-Glc', 'NaN3/D-Glc'). The rates of encystment (%) were measured 8 hr after the onset of induction. (A-2), (B-2), (C-2) The rates of b/a and d/c indicate the rates of encystment suppression mediated by addition of 0.5 mM D-glucose in the absence and presence of ATP synthesis inhibitors, respectively.

attributed to the proliferation of bacteria but occurs due to D-glucose.

In order to know whether *Colpoda* may internalize D-glucose and utilize it as a nutrient source, cell growth was examined in the presence of D-glucose (Fig. 2). Addition of D-glucose to the external medium caused proliferation of *Colpoda* vegetative cells (Fig. 2A-1), accompanied by an increase in cell length and width (Fig. 2B). Here, the question arises as to whether such *Colpoda* growth is possibly due to a proliferation of bacteria (foods for *Colpoda*) evoked by the nutrient supply. A marked growth of *Colpoda*, however, was observed even in the medium containing D-glucose and ampicillin (Fig. 2A-2) in which proliferation of bacteria induced by D-glucose was completely inhibited (Fig. 1C). It

can be concluded, in consequence, that D-glucose is internalized into the *Colpoda* cell interior and utilized as a nutrient source. Then, does D-glucose act on certain receptors on the plasma membrane, or do the internalized molecules or their metabolic products affect intracellular signaling pathways leading to encystment induction? It is known that in *Tetrahymena*, the uptake of D-glucose is inhibited by phlorizin (Aomine, 1974). As shown in Fig. 3A, in the presence of phlorizin (Phz), cell growth induced by D-glucose was suppressed, suggesting that the uptake of D-glucose was inhibited. In such conditions, the encystment-suppressing effect of D-glucose was cancelled (Fig. 3B). These results suggest that internalized D-glucose or its metabolic products may have a suppressing effect on the pathways leading to encystment induction.

We examined final metabolic products of D-glucose on encystment induction, using inhibitors for ATP production such as a kind of protonophore CCCP (an uncoupler of oxidative phosphorylation) (Skulachev, 1998), oligomycin (an inhibitor of ATP synthesis) (Lardy et al., 1964), and sodium azide (an inhibitor of cytochrome c oxidase) (Bennett et al., 1996) (Fig. 4). Concentrations of inhibitors employed in the present assays were the threshold concentrations so as to maintain normal configuration and motility of *Colpoda*. The encystment-suppression rates (b/a) mediated by D-glucose and those (d/c) in the presence of ATP production inhibitors are shown in Fig. 4 (A-2, B-2, C-2). These rates were obtained from left figures (A-1, B-1, C-1). As shown in Fig. 4 (A-2, C-2), the encystment-suppression rates were significantly reduced in the presence of CCCP or NaN_3 and tended to decrease in the presence of oligomycin (Fig. 4, B-2). These results suggest that D-glucose-mediated encystment suppression may be responsible for final metabolic products of D-glucose.

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