

Effects of Salt Concentration and Bacteria on Encystment Induction in Ciliated Protozoan *Colpoda* sp.

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Summary. The promoting or suppressing elements for cyst formation (encystment) of *Colpoda* sp. was examined. Encystment was promoted by an increase in concentration of ions such as Ca^{2+} , Na^+ and K^+ contained in the surrounding medium, which may be detected by ciliates as an environmental signal for forthcoming desiccation, and was suppressed by the presence of bacteria in the surrounding media. When the surrounding media contained both low concentrations (1 mM) of salts and bacteria (10^7 cells/ml), encystment triggered by cations was completely canceled. However, a gradual increase up to finally 8 mM in salt concentration accomplished by a natural evaporation of the surrounding saline solutions invalidated the encystment-suppression effect by bacteria.

Key words: *Colpoda*, encystment, environmental signals, desiccation.

INTRODUCTION

During the processes of cyst formation (encystment) and excystment in protozoans, overall morphogenetic reconstruction of the cell occurs (Grimes 1973, Matsusaka 1979, Delgado *et al.* 1987, Foissner and Foissner 1987, Matsusaka *et al.* 1989, Martín-González *et al.* 1992, Delmonte Corrado *et al.* 1996). These processes are believed to be controlled by unknown intracellular signaling chains leading to gene expression initiated by environmental signals (Hirukawa *et al.*

1998, Suizu and Matsuoka 1998, Villalobo *et al.* 2001), and these processes are excellent model systems for elucidating the molecular mechanism of cellular-level morphogenesis. One of the strategies for the molecular mechanism of the encystment or excystment is to isolate and characterize receptors that are activated by environmental signals. To date, only a certain receptor-like molecule responsible for encystment has been isolated in a soil amoeba and named "encystment-stimulating protein" (ESP) (Yang and Villemez 1994). However, the kind of environmental signal actually activating the receptor (ESP) has not been determined. The environmental signals that directly activate the receptors are still not understood, although in many protozoans, several environmental changes (desiccation, starvation, accumulation of metabolic wastes) inducing encystment or ex-

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cystment have been listed (Rastogi *et al.* 1973, Yonezawa and Takahashi 1990, Gutiérrez *et al.* 2001, Tomaru 2002); for example, in the case of encystment induced by starvation, several factors including (1) discontinuation of nutrient supply, (2) absence of food vacuoles, (3) absence of substances released from bacteria in surrounding medium are candidates for signals to activate certain receptors.

In the case of *Colpoda* employed in the present study, most of the cells suspended in a fresh saline solution (1 mM CaCl₂, 1 mM KCl, 0.1 mM Tris-HCl, pH 7.1) without bacteria are transformed into cysts, although a simple starvation of the cells suspended in 0.1 mM Tris-HCl buffer without any other salts does not induce a prominent cyst formation (Watoh *et al.* 2003). Increased osmolality of the surrounding medium resulting from the addition of 5 mM mannitol (corresponding to the osmolality of the solution containing 1 mM CaCl₂ and 1 mM KCl) does not induce encystment (Watoh *et al.* 2003). *Colpoda* cells that are transferred from culture medium into the saline solution probably detect an increased ionic concentration as a signal for forthcoming desiccation. On the other hand, such an induction of encystment is completely invalidated by the presence of bacteria (10⁷ cells/ml) in the surrounding medium (Watoh *et al.* 2003). A desiccation signal should have precedence over other environmental signals, because desiccation is invariably lethal for vegetative cells. Presumably much higher concentrations of salts are needed to overcome the encystment-suppression effect by bacteria. The present study demonstrated that a gradual concentration of the surrounding media containing salts invalidated the encystment-suppression effect by bacteria.

MATERIALS AND METHODS

Colpoda sp. was isolated from cysts adhered to dried fallen leaves in the field, and cultured in an infusion of dried cereal leaves (0.1 %) inoculated with bacteria (*Enterobacter aerogenes*) at 23°C in the dark. Bacteria were cultured on agar plates containing 1.5% agar, 0.5% polypeptone, 1% meat extract and 0.5% NaCl. Prior to encystment induction, cultured vegetative cells were rinsed 3 times in each test solution by transferring the cells into fresh test solutions using a thin glass pipette, and 50-60 cells were subsequently suspended in 1.5 ml of each test solution. The rate of encystment was expressed as the percentage of total number of tested cells (50-60 cells). Columns and attached bars shown in Figs. 1- 4 correspond to the means of 4 identical measurements (50-60 cells per measurement) and standard errors. Each series of measurements was performed using the cells obtained from each batch culture at about 25°C

under fluorescent room lighting (0.1~ 0.5 W/m²). 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 and the value of optical density at 600 nm (OD₆₀₀).

RESULTS AND DISCUSSION

Encystment induction

Effect of Tris-HCl: When cultured *Colpoda* cells were suspended in saline solution containing 1 mM CaCl₂, 1 mM KCl and 0.1 mM Tris-HCl (pH 7.2), most of the cells encysted. This implies that certain ions contained in this saline solution may have induced encystment. Therefore, we examined first whether Tris-HCl is effective for initiating cyst formation (Fig. 1). When the cells were transferred into 0.1 mM Tris-HCl buffer (pH 7.2), a few of the cells encysted (Fig. 1). Such a slight induction of encystment may not have been due to Tris⁺ or Cl⁻, because some of the cells also encysted even when the cells were suspended in distilled water (Fig. 4d). Although at concentrations of Tris-HCl greater than 1 mM, the mean values of encystment rates tended to increase, there was no significant difference among the 3 series of different concentrations of Tris-HCl ranging from 0.1 mM to 5 mM ($p > 0.05$, Kruskal-Wallis test) (Fig. 1). In consequence, Tris-HCl does not have a prominent effect on the induction of encystment at least below 5 mM.

Effects of low concentrations of Ca²⁺: Among several saline solutions at 1 mM concentration, the solutions containing CaCl₂ showed prominent encystment induction (Fig. 2); in this experiment, only the encystment rate induced in the solution containing CaCl₂ is significantly different from that induced in the medium containing only 0.1 mM Tris-HCl (Fig. 2; see the column labeled "None") ($p < 0.05$, Mann-Whitney test). The fact that 5 mM Tris-HCl buffer (containing 4.5 mM Cl⁻) (Fig. 1) and 1 mM MgCl₂ solution were not effective (Fig. 2) implies that the effect of CaCl₂ may be not attributed to Cl⁻ but to Ca²⁺ at least in these concentrations.

It is possible that encystment induction in some of the cells suspended in Tris-HCl buffer without other any salts or suspended in distilled water is mediated by Ca²⁺ contamination in the solution. If so, the encystment of the cells should be completely suppressed in medium in which free Ca²⁺ is eliminated by EGTA. If the concentration of free Ca²⁺ contaminating the medium is as-

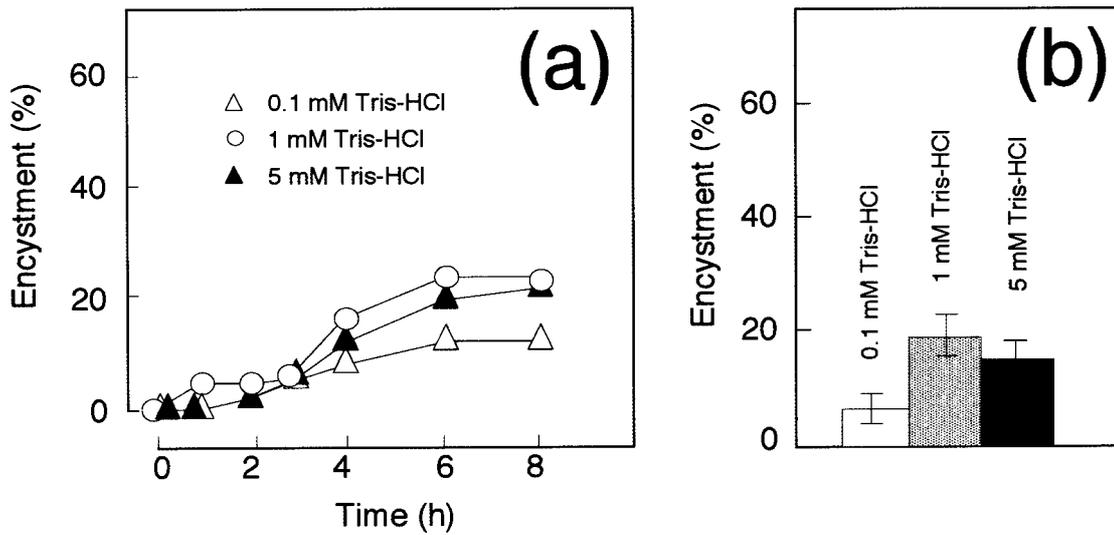


Fig. 1. Effects of various Tris-HCl concentrations on the induction of *Colpoda* encystment. **a** - typical time course of encystment; **b** - the rate of encystment (%) at 8 h after induction.

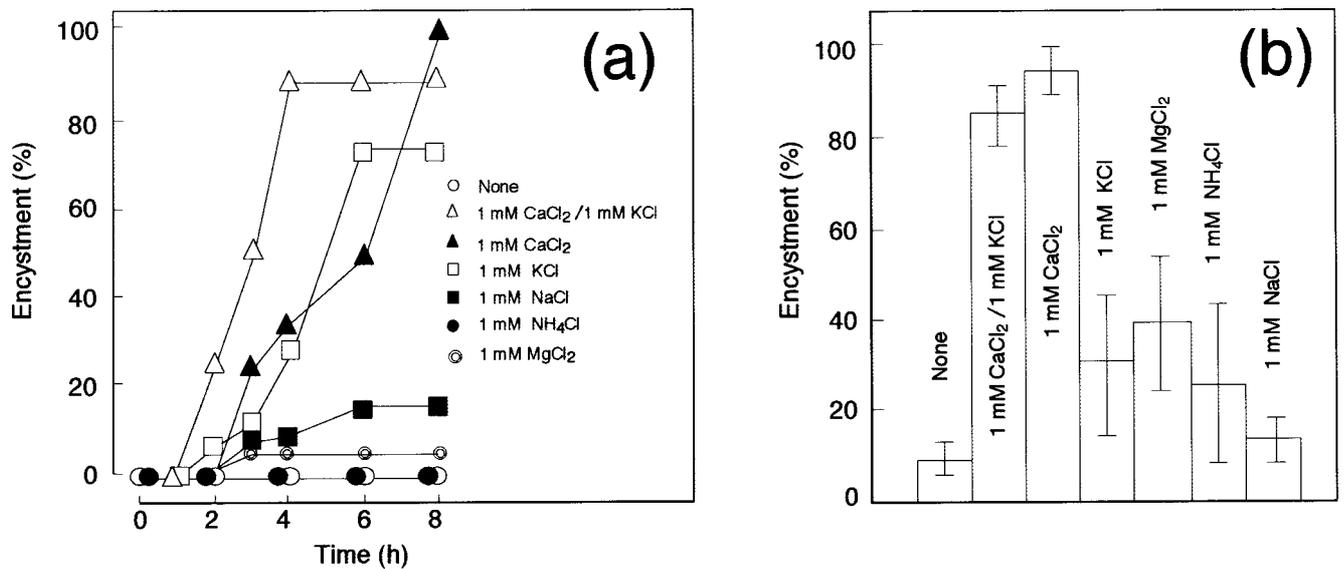


Fig. 2. Effects of various ions (salts) on the induction of *Colpoda* encystment. Every solution contained 0.1 mM Tris-HCl (pH 7.2) and various salts. **a** - typical time course of encystment; **b** - the rate of encystment (%) at 8 h after induction.

sumed to be 10^{-6} M, the addition of 0.1 mM EGTA (final concentration) reduces the free Ca^{2+} concentration to about 10^{-9} M. A few cells encysted in spite of the addition of 0.1 mM EGTA (Fig. 3); there is no significant

difference between Ca^{2+} -free medium (without addition of CaCl_2) and the medium containing EGTA ($p > 0.05$, Mann-Whitney test). It is likely that, in the solution containing no promoting or suppressing factor for en-

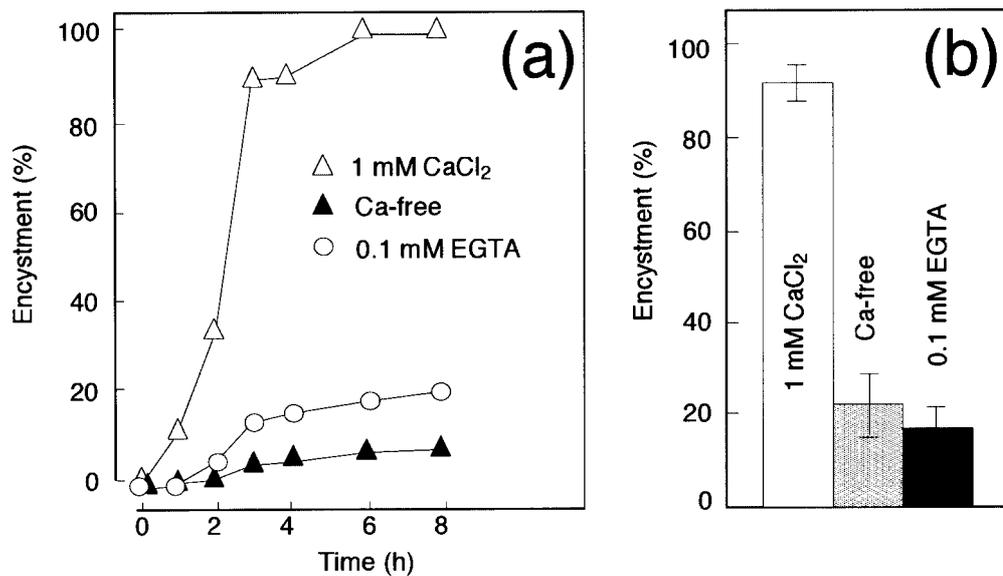


Fig. 3. Effect of elimination of Ca²⁺ contamination in the surrounding medium on encystment of *Colpoda*. Every solution contained 0.1 mM Tris-HCl (pH 7.2). **a** - typical time course of encystment; **b** - the rate of encystment (%) at 8 h after induction.

cystment, the determination of whether encystment is triggered is unsettled.

Encystment induction and suppression

Effects of ions concentrations and bacteria: In the saline solutions containing CaCl₂, KCl or NaCl gradually concentrated up to 8 mM by natural evaporation, encystment was prominently promoted (Fig. 4). The results indicate that *Colpoda* cells detect the rise of concentration of cations or anions in the surrounding medium as the signal to encyst because of forthcoming desiccation. The induction of cyst formation by 1 mM salt concentration was severely repressed by the presence of bacteria (10⁷ cells/ml) in the surrounding medium (Fig. 4); however, a gradual rise in the concentration of CaCl₂ overcame the bacterial effect (Fig. 4a), despite the fact that the bacterial density increased concomitantly with the increase in concentration of the CaCl₂ solution. There was a significant difference between the encystment rate of the cells suspended in the solution containing both 1 mM CaCl₂ and bacteria, and the rate in the concentrated medium (8 mM CaCl₂) with bacteria ($p < 0.05$, Mann-Whitney test). Concentrated NaCl solution also overcame the bacterial effect (Fig. 4c); there was a significant difference between the solution containing both 1 mM NaCl and bacteria, and

the concentrated solution with bacteria ($p < 0.05$, Mann-Whitney test). In the case of KCl solution (Fig. 4b), no marked effect was observed ($p > 0.05$, Mann-Whitney test). As shown in Fig. 4d, the evaporation of distilled water suspending the cells up to 1/8 volume had no effect. This result suggests that the encystment-promoting effect of concentrated NaCl or KCl solution is not attributable to the concentration of contaminated Ca²⁺ in the solutions. In concentrated MgCl₂ solution, a large number of cells were killed.

Tris-HCl buffer at 2.2 mM (pH 7.2) contains 2 mM Cl⁻ (identical molar concentration with Cl⁻ produced by ionization of 1 mM CaCl₂), and the buffer concentrated up to 1/8 volume (17.6 mM Tris-HCl) contains 16 mM Cl⁻. Concentration of Tris-HCl up to 1/8 volume did not have a marked encystment-promoting effect (Fig. 4e). This result suggests that neither Cl⁻ nor Tris⁺ is involved in the promotion of encystment, and that the encystment-promoting effect of salts such as CaCl₂, NaCl or KCl might be attributed to cations produced by ionization of these salts.

Mannitol solution at 5 mM whose osmolality is approximately equivalent to that (5 mOsm) containing 1 mM CaCl₂ and 1 mM KCl did not promote encystment (Watoh *et al.* 2003). In addition, the fact that Tris-HCl buffer concentrated up to 17.6 mM equivalent to *ca* 33.6

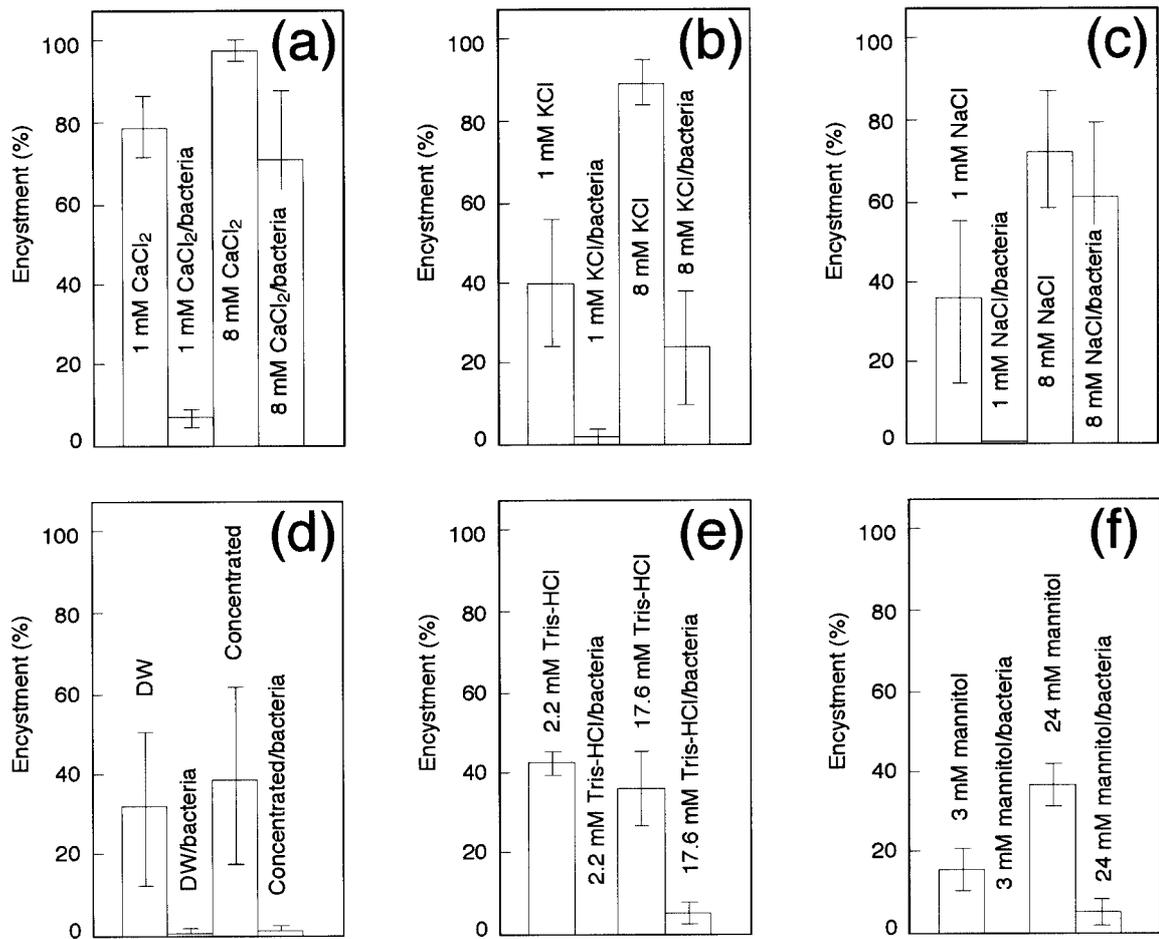


Fig. 4. Encystment-promoting effect by concentrated media and dominance between these effects and the encystment-suppressing effect by bacteria (10^7 cells/ml). The rate of encystment was expressed as the percentage of total number of tested cells (50-60 cells at *ca* 7 h after the *Colpoda* cells were suspended in the solutions. **a-c** - the 1 mM saline solutions; **d** - distilled water (DW); **e** - 2.2 mM Tris-HCl (pH 7.2); **f** - 3 mM mannitol. Solutions with or without bacteria (10^7 cells/ml) were gradually concentrated up to 1/8 volume for *ca* 7 h at room temperature by natural evaporation to finally produce concentrations of 8 mM saline solutions (a-c), 17.6 mM Tris⁺ and 16 mM Cl⁻ (e) and 24 mM mannitol solution (f), respectively. The media did not contain Tris-HCl buffer except for the solution used in Fig. 4 (e).

mOsm does not have an encystment-promoting effect (Fig. 4e) implies that the encystment-promoting effect of concentrated saline solutions may not be responsible for the increased osmolality of the surrounding media. In order to confirm that, we examined the effect of highly concentrated mannitol solution. As shown in Fig. 4f, a gradual concentration of 3 mM mannitol solution to produce a final molar concentration of 24 mM whose osmolality is approximately equivalent to that of 8 mM CaCl₂ solution did not induce a number of cyst formation. However, there was a significant difference between the encystment rate of the cells suspended in 3 mM mannitol and the rate in its concentrated medium

(24 mM mannitol) ($p < 0.05$, Mann-Whitney test). Judging from the statistical analysis, we cannot completely eliminate the effect of osmolality on the promotion of encystment.

The use of some concentrated saline solutions such as CaCl₂ or NaCl solution invalidated the encystment-suppressing effect of bacteria (Figs 4a, c). This is not an issue of the viability of bacteria in such concentrated media, because bacteria were still alive even in the highly concentrated saline solution (Fig. 5); there was no significant difference between low concentration of saline solution (1 mM CaCl₂, 1 mM KCl and 5 mM Tris-HCl, pH 7.2) and highly concentrated solution (50 mM

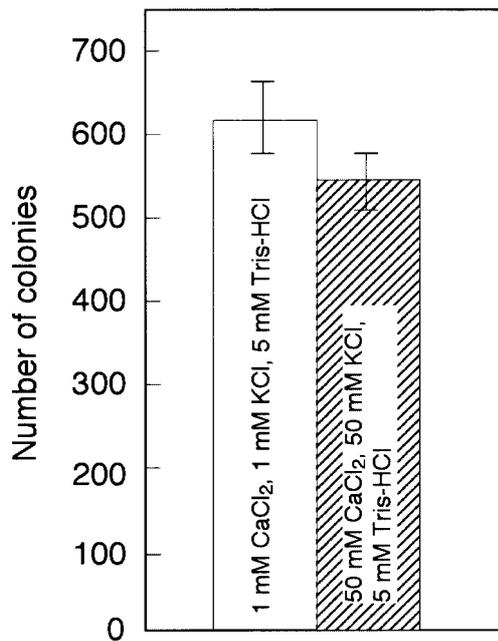


Fig. 5. Effect of concentration of saline solution on viability of bacteria. Living bacteria (10^7 cells/ml) were suspended overnight in saline solution containing 5 mM Tris-HCl (pH 7.2), 1 mM CaCl₂ and 1 mM KCl (open column) or solution containing 5 mM Tris-HCl (pH 7.2), 50 mM CaCl₂ and 50 mM KCl (shaded column). The bacterial suspensions were then 1000-times diluted, and 50 μ l of the suspension was transferred onto the agar plate to spread. After 1-day incubation at room temperature, the colonies of each plate were counted. Columns and attached bars correspond to the means of 4 identical measurements and standard errors.

CaCl₂, 50 mM KCl and 5 mM Tris-HCl, pH 7.2) ($p > 0.05$, Mann-Whitney test).

In conclusion, *Colpoda* detects increased concentration of cations such as Ca²⁺, Na⁺ or K⁺ in the external medium as the environmental signal for forthcoming desiccation and promptly initiates cyst formation, and Ca²⁺ is most effective among these cations. Such a desiccation signal would have precedence over other encystment-suppressing elements derived from bacteria.

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