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Short communication

ATP accumulation in early resting cyst formation towards cryptobiosis in *Colpoda cucullus*

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Abstract. Resting cyst formation is a crucial process of cryptobiosis in protists. In colpodid ciliates, cyst formation is accompanied by large-scale morphological changes such as changes of cell shape, resorption of cilia, and formation of a cyst wall; additionally, the cell cycle is arrested. These changes provide acquired tolerance against environmental stresses. During cyst formation, mitochondrial membrane potential is reduced and the level of the ATP synthase beta chain is suppressed, strongly indicating that metabolism has ceased. Here, however, we show that ATP levels are elevated during the initial phases of encystment implying that metabolism may not be completely suppressed. This finding suggests another aspect of resting cyst formation that is not applicable to cryptobiosis.

Keywords: dormancy, ciliate, metabolism, ATP synthase, mitochondria

INTRODUCTION

Cryptobiosis is a survival response to adverse environmental conditions; organisms that undergo cryptobiosis become metabolically inactive and show no signs of life (Keilin 1959; Cleg 2001). In protists, and especially ciliates, the formation of resting cysts is a feature of cryptobiosis and is well known as a strategy against environmental stresses (Gutiérrez et al. 1990, 2001; Verni and Rosati 2011). Resting cyst formation was an evolutionary breakthrough for protists enabling them to survive in terrestrial environments and in temporary water environments. Resting cyst formation is accompanied by dynamic changes in cellular structures, such as the dedifferentiation or resorption of cilia (Benčaťová and Tirjaková 2017; Li et al. 2017), altered gene expression patterns (Jiang et al. 2019; Pan

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et al. 2019, 2021), and changes in protein levels (Sogame et al. 2012, 2014, 2020; Chen et al. 2014; Gao et al. 2015).

The resting cysts of colpodid ciliates can survive starvation (Gutiérrez et al. 2001), desiccation (Corliss and Esser 1974; Müller et al. 2010), high and low temperatures (Taylor and Stickland 1936), freezing (Uspenskaya and Lozina-Lozinsky 1979; Matsuoka et al. 2020), extreme pH (Sogame et al. 2011; Nakamura et al. 2020), exposure to UV (Matsuoka et al. 2017; Yanase et al. 2020) and gamma radiation (Saito et al. 2020a, b), and electrostatic exposure (Saito et al. 2023). Although resting cyst formation enables survival, the protist loses the ability to move, reproduce, or feed. Metabolic activity is reduced to an undetectable level. During resting cyst formation in *Colpoda cucullus*, the membrane potential of the mitochondria initially ceases, and then the mitochondria are digested (Funatani et al. 2011). In addition, the level of the ATP synthase beta chain also decreases during the early stages of encystment induction (~5h after the induced onset of encystment) (Sogame et al. 2012b, 2014). These events embody the definition of Keilin (1959) and Clegg (2001) that encystment involves cessation of metabolic activity. However, this claim is not a full description of encystment as we showed previously that resting cysts are not completely inactive but can undertake repair of cellular damage caused by gamma irradiation (Sogame et al. 2019; Saito et al. 2020a). Thus, the survival rate of resting cysts exposed to gamma rays is elevated simply through incubation in the cystic state compared to cysts that have not been incubated (Sogame et al. 2019). Furthermore, gamma ray-induced carbonylated proteins are repaired, i.e., the level of protein carbonylation is reduced (Saito et al. 2020a). These results suggest that cysts may maintain some metabolic activity for energy production, albeit without mitochondrial activity. In this study, we provide a possible answer to resolve these contradictions.

MATERIALS AND METHODS

Cells and culture

Colpoda cucullus R2TTYS strain cells were cultured as described by Saito et al. (2023). Encystment of *Colpoda* cells was induced by placing the cells at a high density (10,000–50,000 cells/ml) in encystment-inducing medium: 1 mM Tris-HCl (pH 7.2), 0.1 mM $CaCl_2$.

Microscopy

Mitochondrial membrane potential was visualized using a Mito PT assay kit (Immunochemistry Technologies LLC., California, USA). Cells were collected by centrifugation (1,500 g for 1 min) and washed twice in 1 mM Tris-HCl (pH 7.2). Mito PT staining was performed using the manufacturer's protocol. After staining, cells were washed twice in each medium and incubated for encystment induction. The stained cells were visualized by fluorescence microscopy using an Axioscope A1 system (Carl Zeiss Japan) with a 475 nm LED laser.

Measurement of the relative amount of ATP

We used the BacTiter-Glo Microbial Cell Viability Assay kit (Promega Corporation, Madison, USA) to compare the levels of ATP in *Colpoda* vegetative cells and cells induced to encyst. Cells were collected and washed by centrifugation (1500 g for 1 min), washed twice in 1 mM Tris-HCl (pH 7.2), and suspended at a high cell density. Ampicillin (final conc. 50 mg/mL) was added for suppression of bacterial proliferation. Calcium ($CaCl_2$: 0.1mM final conc.) was also added for induction of encystment. Immediately or after encystment induction, cells were collected and frozen in liquid nitrogen, and stored at –80°C. The frozen cells were thawed and homogenized on ice and then disrupted using an MS-100R beads crusher (Tomy Digital Biology Co., LTD., Tokyo, Japan). The relative amount of ATP in 10,000 cells was determined using the BacTiter-Glo Microbial Cell Viability Assay kit and measured using a luminometer (Promega) as described in the manufacturer's protocol.

Real time PCR analysis

Total RNA was extracted using the TRI reagent (Molecular Research Center, Inc., Cincinnati, USA) from 500,000 vegetative cells and from 500,000 cysts at 1, 3, 5, and 12 h after induction of encystment. The extracted total RNA was purified using the Direct-zol RNA purification system (Zymo Research Crop., California, USA) following the manufacturer's protocol. Reverse transcription was performed using a Transcriptor First Strand cDNA synthesis Kit (Roche Diagnostics, Mannheim, Germany) following the manufacturer's protocol. Gene-specific primers for the ATP synthase beta subunit and the transcription elongation factor SPT4 (internal control) were designed using Primer3 software (https://www.bioinformatics.nl/cgi-bin/primer3plus/primer3plus.cgi) and the gene sequences were used have been registered by Suzuki and Sogame in the DDBJ database (DRR275117-DRR275118; Suzuki and Sogame, DDBJ/ unpublished, Saito et al., in preparation). The primer sequences were as follows.

ATP synthase beta subunit
sense: 5'-GGGAGGAAAGATCTCCCAAG-3'
anti-sense: 5'-CCACCAGTGTCAACGACATC-3'
transcription elongation factor SPT4
sense: 5'- TCCTTCGAAGCTTGGTGTTG-3'
anti-sense: 5'- ATGCCTTACATGCCGTCTTG-3'

All reactions for the real time PCR analysis were performed using Real time PCR system STEP1 (Thermo Fisher Scientific K.K., Tokyo, Japan) and Power up SYBER (Thermo Fisher Scientific) with three technical replicates. The amplification cycle was: 50°C for 2 min. and 95°C for 2 min.; 40 cycles at 95°C for 15 sec., 55°C for 15 sec., and 72°C for 1min. Melt curve readings were obtained using the manufacturer's protocol. All data were an-

alyzed by the ΔΔct method using Real-Time PCR System Software (Thermo Fisher Scientific).

Statistical analyses

The statistical analyses, Tukey's test for Fig. 2 and Mann-Whitney U test for Fig. 3, were performed using Bell Curve for Excel software (Social Survey Research Information Co., Ltd., Tokyo, Japan).

RESULTS AND DISCUSSION

Following the induction of encystment, *Colpoda* vegetative cells become rounder and cease movement within 1 h (Asami et al. 2010; Fig. 1A). The vegetative cells and early stage encysting cysts had an active mitochondrial membrane potential but this was diminished at 3 h after encystment induction and could not be detected at 5 h after induction (Fig. 1B). The loss of mitochondrial membrane potential has already been reported in previous studies (Funatani et al. 2010; Sogame et al. 2014), but was performed again so that the entire sample cell population could be confirmed. These results show similar trends to those previous reports by Funatani et al. (2010) and Sogame et al. (2014). In addition, some mitochondria had been digested at 1 h after encystment induction and small mitochondria lacking a membrane potential appeared; the role of these small mitochondria and the reason for their appearance are unclear (Funatani et al. 2010). The protein level of ATP synthase beta chain, which catalyzes ATP formation in eukaryotic cells (Huen et al. 2010), is decreased during *C. cucullus* encystment (Sogame et al. 2012, 2014), and similarly in *Euplotes* encystment (Chen et al. 2018), indicating a reduction in metabolic activity. Expression of the gene for ATP synthase beta chain was also suppressed within 1 h of encystment induction (Fig. 2), which strongly indicates that mitochondrion-mediated

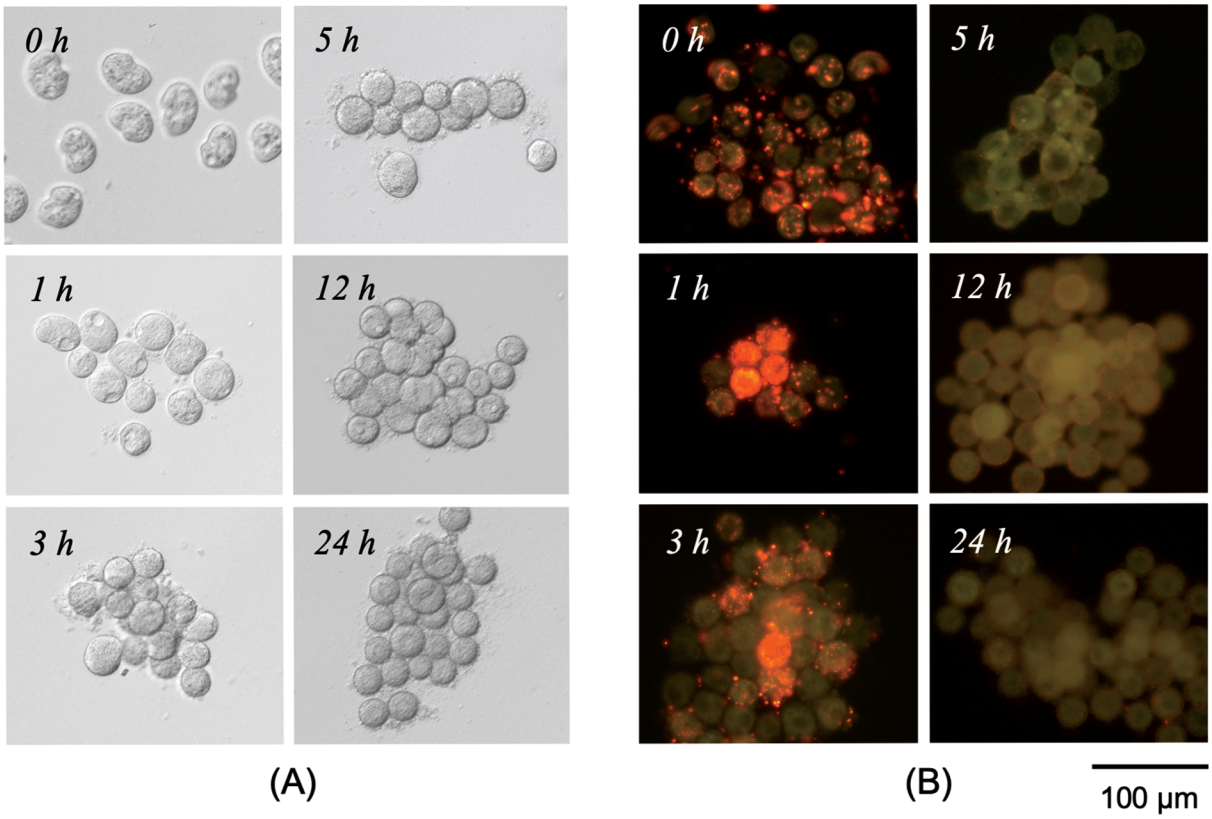

Fig. 1. Visualization of the mitochondrial membrane potential of *Colpoda* vegetative cells using a Mito PT assay kit; the results from cells at 0 h and cells at 1–24 h after the onset of encystment induction are shown. Differential interference microscopic observation (A) and fluorescence microscopic observation (B). Cells that contain mitochondria with polarized inner membranes show orange fluorescence, whereas those with depolarized mitochondria show green fluorescence. The bar represents 100 µm.

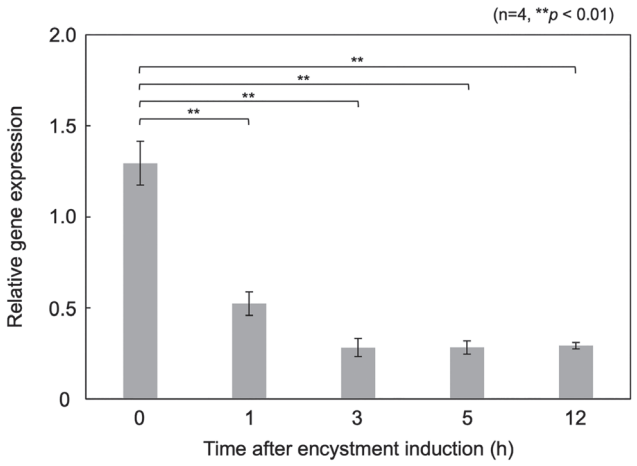

Fig. 2. Relative gene expression of ATP synthase beta chain by real time PCR analysis. The columns and attached bars represent the means and standard errors of 4 identical replicates, respectively. Double asterisks represent significant differences at $p < 0.01$.

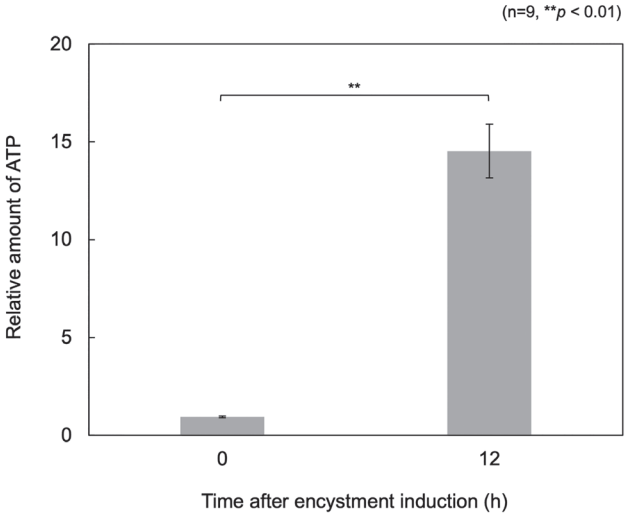

Fig. 3. Measurement of the relative amount of ATP per 10,000 cells in vegetative cells and cells at 12 h after the onset of encystment induction. The columns and attached bars represent the means and standard errors of 9 identical replicates, respectively. Double asterisks represent significant differences at $p < 0.01$.

metabolic activity ceased during encystment. In addition, enzymes related to the TCA cycle and oxidative phosphorylation glycolysis/gluconeogenesis are reduced during encystment (Chen et al. 2018; Jiang et al. 2019; Pan et al. 2019). These results indicate that biosynthetic activity is suppressed during encystment, leading to the down-regulation of metabolic activity (Li et al. 2022).

In a previous study, we unexpectedly found that cellular damage could be repaired in resting cysts, and that this characteristic resulted in an elevated survival rate in gamma-irradiated cysts (Sogame et al. 2019); the level of protein carbonylation was also reduced by incubating irradiated cells as cysts (Saito et al. 2020a). These results imply that metabolic and biosynthetic activities can occur in resting cysts, despite the fact that the mitochondria have lost their membrane potential. The results of this study are consistent with our observation in previous studies (Sogame et al. 2019; Saito et al. 2020a). The relative amount of ATP in resting cysts at 12 h after the onset of encystment induction was approximately 14.5 times higher (range 6.5 to 22.0) than that of vegetative cells (Fig. 3), although the mitochondrial membrane potential had stopped at that time (Fig. 1).

It is widely believed that resting cysts during cryptobiosis do not have any measurable metabolic activity (Gutiérrez et al. 1990, 2001; Verni and Rosati 2011). Various analyses have demonstrated that the mitochondria are inactive (Funatani et al. 2010, Sogame et al. 2014) and that metabolism in resting cysts has ceased (Gutiérrez et al. 1990, 2001). Here, however, we showed that ATP accumulation could occur during resting cyst formation, despite the silencing of mitochondrial metabolic activity. These results suggest that the main metabolic process of resting cysts switches to a metabolic process distinct from oxidative phosphorylation in vegetative cells. Perhaps some other metabolic processes such as lactic acid fermentation or alcohol fermentation, may be activated in resting cysts as has been reported in *Entamoeba histolytica* (Montalvo et al. 1971). At the moment, it is not possible to completely rule out a simple accumulation of ATP due to the decrease in ATP consumption. Detailed metabolite and metabolome analyses will be required to determine whether mitochondria-dependent oxidative phosphorylation is completely stopped and other metabolic systems are activated. This study shows that resting cysts, which had been believed to have ceased metabolism, may be undergoing different metabolic processes from those in vegetative cells. This difference may contribute to the repair of cell damage by environmental stresses in resting cysts. Resting cysts, when not in the state of cryptobiosis, may be in an entirely different physiological condition that has metabolic activities that differ from vegetative cells.

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