J. Protozool. Res. 30. 38-46 (2020)

Tolerance of *Colpoda cucullus* Nag-1 wet resting cysts to extreme pH (pH 1 and 13): Implications of less permeability of the cyst membrane to H^+ and OH^-

Rikiya Nakamura[1,2], Yoichiro Sogame[3], Mikihiko Arikawa[4], Futoshi Suizu[5,*], Tatsuomi Matsuoka[4,*]

[1] Department of Biological Science, Faculty of Science, Kochi University, Kochi 780-8520, Japan
[2] Chikazawa Paper Co., Ltd., Japan
[3] Department of Applied Chemistry & Biochemistry, National Institute of Technology, Fukushima College, Iwaki, Fukushima, 970-8034, Japan
[4] Department of Biological Science, Faculty of Science and Technology, Kochi University, Kochi 780-8520, Japan
[5] Division of Cancer Biology, Institute for Genetic Medicine, Hokkaido University, Sapporo 060-0815, Japan
*Corresponding authors:
Tatsuomi Matsuoka; E-mail: tmatsuok@kochi-u.ac.jp
Futoshi Suizu; E-mail: f_suizu@igm.hokudai.ac.jp

ABSTRACT

In parasitic unicellular eukaryotes, the tolerance to the pH (1-1.5) of gastric acid is a crucial survival strategy so that they can proliferate in the intestinal tract. We found that the resting cysts of non-parasitic soil ciliate *Colpoda cucullus* Nag-1 showed a strong tolerance to both extremely low and high pH. The purpose of this study was to explore the tolerance mechanism to extreme pH. Most cysts were alive after exposure to 0.1 M HCl (pH 1) for 4 h, or after exposure to 0.1 M NaOH (pH 13) for 3 h. Such tolerance to extreme pH is acquired gradually over several days after encystment induction. The resting cysts were reversibly dehydrated by osmotic pressure when they were transferred from water to 0.1 M HCl or 0.1 M NaOH. This result suggests that H^+/Cl^- and Na^+/OH^- may diffuse through the cyst wall to reach the plasma membrane. Acid tolerance was reduced in the presence of protonophore (CCCP), suggesting that less permeability of the cyst plasma membrane to H^+ may be responsible for acid tolerance.

Keywords: *Colpoda*; resting cysts; tolerance to extreme pH

INTRODUCTION

The formation of dormant forms or resting cysts tolerant to desiccation (Corliss and Esser, 1974), low temperature (Taylor and Strickland, 1936) or UV rays (Lonnen, *et al*., 2014; Matsuoka *et al*., 2017; Yamane *et al*., 2020) is an adaptive strategy for the survival of unicellular eukaryotes in terrestrial environments. Vegetative forms of terrestrial unicellular

eukaryotes such as the ciliate *Colpoda* promptly transform into resting cysts when they detect approaching desiccation. When favorable aquatic environments are recovered, they excyst to emerge from the resting cysts, and grow rapidly.

When *Colpoda cucullus* Nag-1 vegetative cells are induced to encyst by being suspended in a Ca^{2+}-containing medium at high cell density (Matsuoka *et al*., 2009), the cells become rounded within 2-3 h, and mucus is expelled into the extracellular space. Subsequently, small sticky globules called lepidosomes (Foissner *et al*., 2011) are extruded, trapped in a mucus layer, and form a mucous/lepidosome layer (Funatani *et al*., 2010; Funadani *et al*., 2016). The spherical cells are surrounded by a single rigid layer (ectocyst layer) in about 3 h, followed by the formation of the first-synthesized endocyst layer just beneath the ectocyst layer. Then, the mitochondrial membrane potential disappears (arrest of mitochondrial activity) (Funatani *et al*., 2010; Sogame *et al*., 2014), and autophagosomes form to digest cellular components of the vegetative cell at 3-5 h. The autophagy continues up to 24 h after encystment induction. The endocyst layers continue to form, and several layers are formed over several days, thereby creating a cyst wall composed of a mucus/lepidosome layer, ectocyst layer and endocyst layers (from outside) (Funatani *et al*., 2010). Many presumed carbohydrate grains accumulate in the cytoplasm over several days, and cilia and kinetosomal structures disappear in mature (over 1-week-old) cysts (Funatani *et al*. 2010).

We previously reported that the wet resting cysts of *C. cucullus* Nag-1 show a tolerance to HCl; most cysts survive in 0.1 M HCl (pH 1), for at least 1 h (Sogame *et al*., 2011). The tolerance to extremely low pH is a crucial survival strategy for parasitic unicellular eukaryotes in the stomach acidic pH (pH 1-1.5) of host animals, so that they can proliferate in the intestinal tract. For example, the vegetative forms (trophozoite) of the free-living pathogenic *Entamoeba histolytica* transform into cystic or infective form, which can tolerate the stomach acidic environment, and excysts in the terminal ileum (Serrano-Luna *et al*., 2013). The cysts of parasitic unicellular eukaryote *Giardia lamblia* also pass through stomach gastric acid alive (Hawrelak, 2003). *Acanthamoeba* that infects the cornea of the eye to cause *Acanthamoeba* keratitis is also resistant against 80 mM HCl, even though it does not infect the gastrointestinal tract (Lloyd, 2014). Whether parasitic or free-living, acid tolerance seems to be a common strategy of unicellular eukaryotes for survival in host animal intestines.

In the present study, we found that wet resting cysts of *C. cucullus* Nag-1 survived at least 3-4 h when they were exposed to both extremely low pH (pH 1) and high pH (pH 13). We discuss the tolerance mechanism of *Colpoda* resting cysts against extreme pH.

MATERIALS AND METHODS

Cell culture and encystment induction

Vegetative cells of *Colpoda cucullus* Nag-1 (Funadani *et al*., 2016) (18S ribosomal RNA gene: GenBank Accession No. AB918716) were cultured in a 0.05% (w/v) dried wheat leaves infusion. Two-day cultured vegetative cells were collected by centrifugation (1,500 × *g*, 2 min), and then suspended at a high cell density (> 5, 000 cells/mL) in an encystment-

inducing medium containing 0.1 mM $CaCl_2$ and 1 mM Tris-HCl (pH 7.2). The cell suspension (approximately 1,000 cells in 200 μL suspension) was dispensed in watch glasses under humid conditions. The resting cysts adhered to the bottom of the watch glass.

Treatment with HCl, NaOH and NaCl solutions

Prior to treatment with test solutions, the encystment-inducing medium in the cyst-adhered watch glass was discarded, washed with water 2-3 times, and then the watch glass was refilled with 300 μL of test solutions. After a certain treatment time, the test solutions were again discarded, washed with running water for 10 min, and a fresh 0.05% wheat leaves infusion was poured in to induce excystment (Watoh *et al*., 2003; Tsutsumi *et al*., 2004). Cysts were randomly chosen, and the number of vacant cysts from which vegetative cells had emerged was counted 24 h after excystment induction. The rates of excystment (Figs. 1, 2, 4) and viability (%) of vegetative cells (Fig. 2) were expressed as the percentage of the total number of observed cells (50 cells). Points and attached bars correspond to the means of 5 (Figs. 1, 2) or 6 (Fig. 4) measurements and their standard errors (SE), respectively. In each measurement set (HCl, NaOH and NaCl in Figs. 1, 2) and each series of measurement (HCl/CCCPs or NaOH/CCCPs in Fig. 4), the same lot samples were used. In Fig. 3, statistical analysis was performed using t-test. *P* values < 0.01 were considered significant.

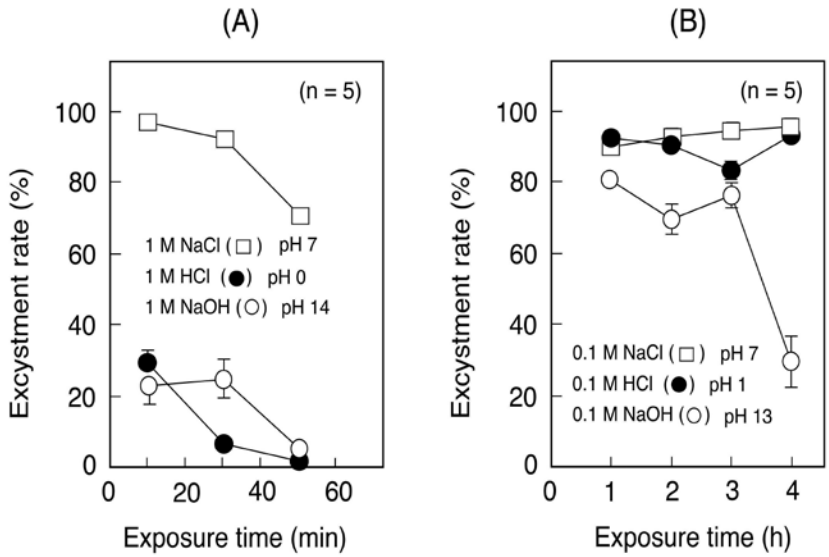

Fig. 1. Tolerance of mature (1-week-old) resting cysts of *C. cucullus* Nag-1 to 1 M (A) and 0.1 M (B) HCl and NaOH test solutions. Ordinate and abscissa indicate excystment rates (%) and the time cysts were exposed to the test solutions.

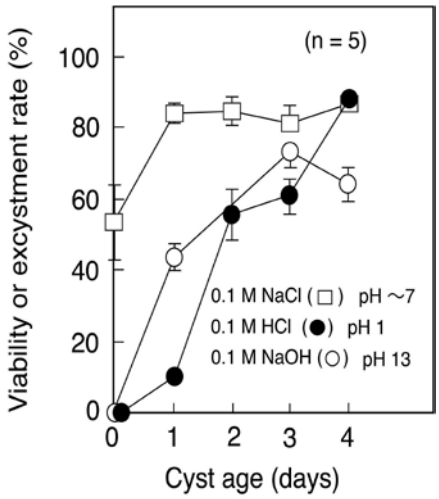

Fig. 2. Tolerance of *C. cucullus* Nag-1 encysting cells at various ages to 0.1 M HCl and 0.1 M NaOH test solutions. Ordinate indicates excystment rates (%) of immature cysts age 1 day or more or viability (%) of vegetative cells that were exposed to the test solutions for 1 h. Abscissa indicates the cyst age of encysting cells. Cyst age '0 day' is the vegetative cells first suspended in encystment-inducing medium and subsequently in the test solutions. In the measurements of viability of vegetative cells, a single lot of 50-cell samples of vegetative cells was prepared using a thin glass pipette.

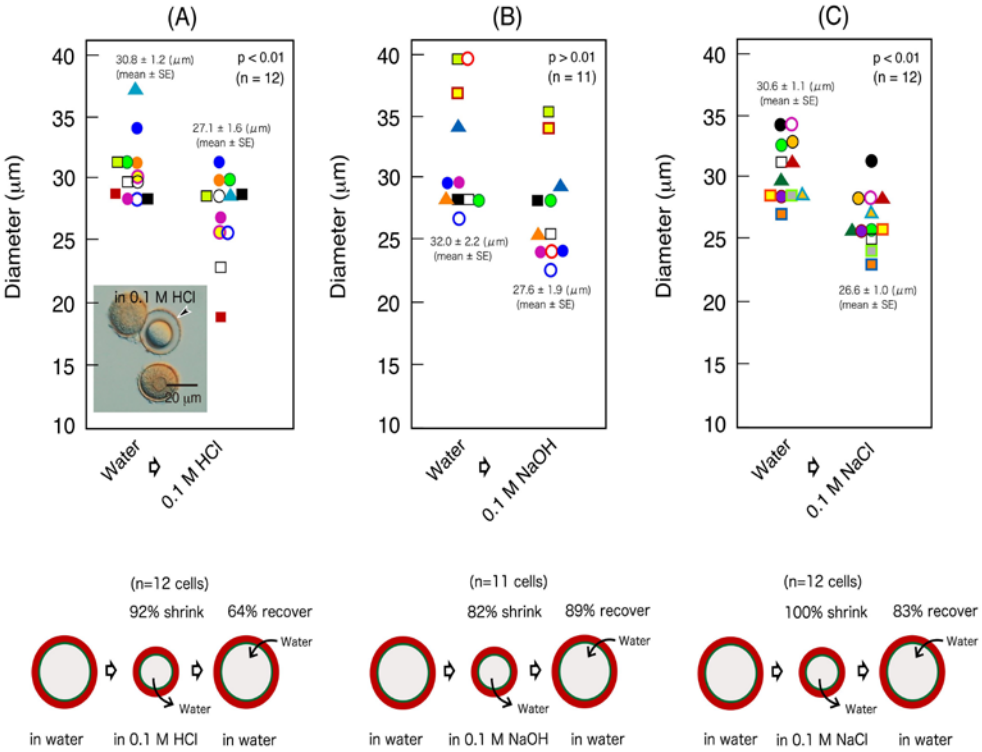

Fig. 3. Osmotic shrinking of *C. cucullus* Nag-1 wet resting cysts (1-week-old) under hypertonic condition caused by 0.1 M HCl (A); 0.1 M NaOH (B); and 0.1 M NaCl (C). The resting cysts adhered to the bottom of watch glasses were first immersed in water for 5 min to measure their diameter (left points in each figure). Thereafter, water was replaced by 0.1 M of each test solution (HCl, NaOH and NaCl), and diameter of the cyst was measured after 5 min (right points in each figure). Each point in each figure represents the diameter of each cyst's cell body, and a set of data markers with the same color and shape indicates the values obtained from the same cyst. One run of measurement in each experiment [(A) water → HCl, (B) water → NaOH, (C) water → NaCl] was done in the same cell. (A) Inset photograph. The cysts immersed for 5 min in 0.1 M HCl, and then returned to water for 5 min. In the cyst indicated by the arrowhead, cell body remains shrunk and detached from cyst wall, which is killed.

In the schematic diagrams, the 'shrink or recover (%)' indicates the percentage of the cysts which shrank by at least 10% in diameter after soaking for 5 min in the test solutions (0. 1 M HCl, 0.1 M NaOH and 0.1 M NaCl), or which recovered by at least 90% after soaking for 5 min in water. The values are expressed as the percentage of total number of observed cells (11-12 cells). One run of measurement (water → test solutions → water) was done in the same cell.

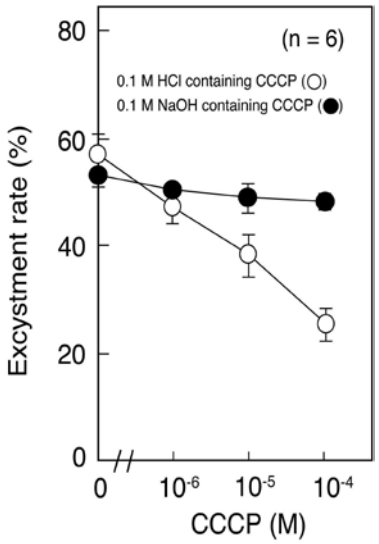

Fig. 4. Tolerance of mature (1- to 2-week-old) resting cysts of *C. cucullus* Nag-1 to 0.1 M HCl (open circles) and 0.1 M NaOH (closed circles) solutions containing various concentrations of CCCP and 0.1% DMSO. The cysts were exposed to each test solution for 1 h.

Protonophore treatment

Carbonyl cyanide 3-chlorophenylhydrazone (CCCP; Wako Pure Chemical Industries) was dissolved in dimethyl sulfoxide (DMSO) to give 100, 10 and 1 μM stock solutions, and 1 mL of each stock solution was added to 1 mL volumes of test solutions to produce final concentrations of test solutions of 10^{-4} M, 10^{-5} M and 10^{-6} M, respectively (final concentration of DMSO at 0.1%).

RESULTS AND DISCUSSION

When mature (1-week-old) resting cysts of *C. cucullus* Nag-1 were exposed to 1 M HCl (pH 0) or 1 M NaOH (pH 14) solution for 10-30 min, 20–30% of the cysts survived (Fig.

1A). When the cysts were exposed to 0.1 M HCl (pH 1) and 0.1 M NaOH (pH 13), most cysts were alive at least for 4 h and 3 h, respectively (Fig. 1B). Such tolerance to extreme pH is gradually acquired over several days after encystment induction (Fig. 2).

In a previous study (Sogame *et al*., 2011), we suggested that the tolerance of *Colpoda* cysts to HCl was possibly acquired by preventing its diffusion across the endocyst layers. If H^+/Cl^- and Na^+/OH^- could diffuse through the cyst wall to reach the plasma membrane, it would be reasonable to expect the cell body of cysts to be dehydrated by osmotic pressure, because the osmolality of the cytosol of *C. cucullus* Nag-1 wet resting cysts is estimated to be below 0.052 Osm/l (Matsuoka *et al*., in press). Actually, the diameter of resting cysts transferred from water into 0.1 M HCl (Fig. 3A) or 0.1 M NaCl (Fig. 3C) decreased significantly (t-test; $p < 0.01$). When the cysts were exposed to 0.1 M NaOH, their mean diameter was reduced (Fig. 3B), although there was not a significant difference between the columns labeled with 'water' and with '0. 1 M NaOH' (t-test; $p > 0.01$). A large number of cysts were reversibly dehydrated, as shown in the schematic drawings in Fig. 3. On the other hand, some of the cysts that had been exposed to HCl or NaOH, and then returned to water remained shrunk. In some cases, the cell body was detached from the cyst wall as shown in the inset photo in Fig. 3A (arrowhead). In this case, selective permeability of the plasma membrane may be destroyed. These results suggest that H^+/Cl^- and Na^+/OH^- may diffuse through the cyst wall to affect the plasma membrane.

The membrane conductance of H^+/OH^- ($G_{H/OH}$) through the phosphatidylethanolamine planner membrane was only elevated 4-fold when H^+ concentration increased from 10^{-7} M to about 10^{-1} M (Gutknecht, 1987). This indicates that the lipid bilayer may not be destroyed even under extreme pH. In acidophiles, one adaptation strategy against extreme acidic pH is impermeability of the cell membrane, which restricts the influx of H^+ into the cytoplasm (Mirete *et al*., 2017). Judging from these previous findings, it is likely that less permeability to H^+ and OH^- may be responsible for the tolerance of *C. cucullus* Nag-1 resting cysts to extreme pH. Actually, our preliminary measurements by means of BCECF-AM [(2',7'-bis-(2-carboxyethyl)-5-(and-6)-carboxyfluorescein acetoxymethyl ester)], fluorescent probe that enables monitoring of cellular pH showed that intracellular pH of *Colpoda* cysts suspended in 0.1 M HCl solution dropped to only 5.8.

If the restriction of permeability of *C. cucullus* Nag-1 cyst membrane to H^+ were responsible for acid tolerance, an elevation of H^+ permeability of the resting cysts by the addition of protonophore (CCCP) could be expected to lose tolerance to extreme pH. Actually, acid tolerance was reduced in a CCCP-concentration dependent manner when the mature cysts were exposed to 0.1 M HCl for 1 h in the presence of CCCP (Fig. 4). On the other hand, the tolerance of cysts to 0.1 M NaOH was not diminished in the presence of CCCP (Fig. 4).

When the cysts were immersed in 0.1 M NaOH solution containing CCCP, H^+ could diffuse from the cell interior to extracellular space by the action of H^+ carrier CCCP, and it is expected that intracellular H^+ concentration drops while OH^- is elevated by the dissociation of intracellular H_2O to keep the ionic product of water at 10^{-14} $(mol/L)^2$. One of the reasons why cysts' tolerance to 0.1 M NaOH was not reduced is that the H^+ carrier CCCP may barely diffuse into the cell interior. Even if CCCP were to sufficiently diffuse into the cell interior,

H^+-transport efficiency from cell interior to exterior may be lower compared to the opposite (from exterior to interior), because the association probability of CCCP and H^+ in cytosol (10^{-7} M H^+) is 10^6-fold lower than that in the surrounding medium (10^{-1} M H^+).

As shown in Fig. 1B, the tolerance of mature cysts to 0.1 M NaOH was suddenly reduced at 4 h. In this case, an inflow of OH^- may have caused the elevation of intracellular OH^- concentration and reduction of H^+, because conductance of artificial membrane to OH^- increases in the high pH range (> pH 11) (Gutknecht and Walter, 1981).

Even in the presence of 10^{-4} M CCCP and 0.1 M HCl, some cysts survived (Fig. 4). This implies that the cyst plasma membrane is still less permeable to H^+ even in the presence of CCCP. Otherwise, the acid tolerance of the *C. cucullus* cysts might be partly attributed to intracellular buffering capacity.

The lower permeability of the *Colpoda* cyst membrane to H^+ or OH^- might be attributed to the absence of an ion channel on the plasma membrane due to the silencing of most genes expressed in vegetative cells. This is because an abrupt influx or efflux of H^+ or OH^- possibly occurs through denatured ion channels or water channels under extreme pH environments. When *C. cucullus* Nag-1 vegetative cells are induced to encyst, the amount of total mRNA contained in the cells abruptly decreases within 3 h to reach about 25% of the initial level at 5 h after onset of encystment. The amount of total proteins begins to decrease in 12 h, and reaches about 25% 1 day after encystment induction (Sogame *et al*., 2014). *Colpoda* cysts begin to acquire tolerance to extreme pH within 1 day of the start of encystment (Fig. 2). These results are not inconsistent with above-mentioned idea.

We note here that there is an association between the lipid composition of the plasma membrane and acid tolerance in some archaea (Mirete *et al*., 2017). During encystment of the unicellular parasite *Giardia lamblia*, lipid composition is altered (Ellis *et al*. 1996). It is also likely that lipid composition of the plasma membrane of *C. cucullus* Nag-1 cysts may be altered, so that membrane permeability to H^+ and OH^- may drop extensively.

The tolerance of *C. cucullus* Nag-1 against 0.1 M HCl implies that this organism may survive in the gastric juices of animals, and may enable cysts to survive in the gastrointestinal tract. It is likely that the acid tolerance of *C. cucullus* Nag-1 may be related to their widespread distribution through animals, as already suggested by Sogame *et al*. (2011).

CONFLICT OF INTEREST

The authors declare that they have no conflict of interest.

SUBMISSION DECLARATION AND VERIFICATION

The authors declare that this manuscript is original, has not been published before and is not currently being considered for publication elsewhere.

ACKNOWLEDGMENTS

We thank Mr. S. Sadano for collaboration on parts of this work. This research was financially supported by a Grant-in-Aid for Scientific Research (B) (#19H03447).

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