Journal of Applied and Natural Science
14(3), 711 - 719 (2022)
ISSN : 0974-9411 (Print), 2231-5209 (Online)
journals.ansfoundation.org

Research Article

A novel phenomenon of pseudoencystment in free living ciliate *Pseudourostyla levis* (Ciliophora, Hypotrichia) from River Yamuna, Delhi

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Article Info
https://doi.org/10.31018/jans.v14i3.3500
Received: May 14, 2022
Revised: July 15, 2022
Accepted: July 20, 2022

How to Cite
Gupta, S. (2022). A novel phenomenon of pseudoencystment in free living ciliate *Pseudourostyla levis* (Ciliophora, Hypotrichia) from River Yamuna, Delhi . *Journal of Applied and Natural Science*, 14(3), 711 - 719. https://doi.org/10.31018/jans.v14i3.3500

Abstract
Free living ciliates are exposed to environmental challenges such as starvation, temperature fluctuations, high population density and salinity variations. Ciliates lower their metabolic activity and form cysts, an adaptive strategy evolved in response to environmental stress. In the present study on *Pseudourostyla levis,* a novel phenomenon of pseudoencystment had been noticed in which cells entered a state of dormancy but did not secrete a cyst wall. The process of pseudoencystment, which physiologically resembled true encystment, was functionally unrelated to it and was thus designated as pseudoencystment. Unlike true cysts, pseudocysts remained dormant for about 12 hours and then reverted to the active trophic state without any change in the environmental conditions. Another unique feature of pseudoencystment, was the synchronized induction of pseudocysts formation, achieved by a brief spell of starvation and the pseudocysts reverted to active trophic state without any change in environmental conditions. The state of dormancy did not provide any long-term protection to the ciliate and appeared to be a short term adaptation to the environment. The present study aimed to analyze the parameters for the induction of pseudocyst formation, cortical topological changes and to study the possible significance of this process. However, occasionally true cysts were also obtained by prolonged starvation, lasting 3-4 days but true cysts required either food or a fresh culture medium for excystation.

Keywords: Adaptive strategy, Environmental challenges, Pseudoencystment, *Pseudourostyla levis*

INTRODUCTION

Ciliates occupy important trophic levels in the food chain (Kaur *et al*., 2019; Chi *et al.,*2021; Matsuoka *et al*.,2021; Pan *et al*., 2021; Li *et al*., 2022; Zhang and Vd'ačný, 2022). Free living ciliates are exposed to several biotic and abiotic environmental stress factors, resulting in homeostatic disturbance (Slaveykova *et al*., 2016). The abiotic stress factors include temperature, pH, radiations, metal toxicity, metal-containing nanoparticles, etc., whereas biotic stress includes parasitic and symbiotic interactions, predator-prey relationships (Slaveykova *et al*., 2016). The ability to respond to unfavourable environmental conditions is crucial for the survival of any organism (Corliss and Esser,1974). Ciliates form resting cysts as an advanced survival strategy to overcome environmental stress, viz., temperature (Matsuoka *et al*., 1990; Maeda *et al*., 2005; Shimada *et al*., 2021), starvation (Gutierrez and Gonzalez, 2002), ultraviolet radiations (Matsuoka *et al*., 2017 and Yamane *et al*., 2020), salinity (Li *et al*., 2017), desiccation (Müller *et al*., 2010 and Benčaťová *et al.,*, 2016).

Under unfavourable conditions of food depletion, ciliates resort to different strategies viz., sexual reproduction, cell reorganization, cannibalism and encystment (Rosati *et al*.,1981; Verni *et al*., 1984; Gutiérrez *et al.,* 2001; Verni and Rosati, 2011; Li *et al*., 2017 ; Chen *et al*., 2018; Pan *et al.,* 2019; Benčat´ova *et al*.,2021; Shimada *et al*., 2021). Encystment is the most common adaption strategy, which apart from protection, also helps in the dispersal of species. Encysted cells are dormant with no visible activity, secrete a thick wall and revert to vegetative proliferative state under favourable conditions. Such cysts have commonly been referred to as 'protective cysts' or 'resting cysts' (Corliss and Esser,1974). Encystment also occurs as an essential part of many ciliates' reproduction cycle, e.g.,*Colpoda* (Burt *et al*., 1941 and Frenkel, 1980), *Tillina magna* (Beers, 1946) and *Paraholosticha sterkii* (Dieckmenn,1988). Such cysts are termed as 'reproductive cysts'. Cyst formation is mainly characterized by drastic cytoplasmic dehydration, which induces decreased metabolic rate and increased autophagy (Gutierrez *et al*., 2001). Although studies have been conducted on the stress

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response of ciliates against biotic and abiotic factors and some molecular and genetic studies, have been conducted but the studies on the process of encystment are still in their infancy. Cyst formation in ciliates provides a model system for studying cell differentiation as the process of encystment and excystment involves dedifferentiation and redifferentiation of cortical structures (Grimes, 1973; Walker *et al*., 1975,; Walker and Maugel, 1980; Li *et al*., 2017; Wang *et al.,* 2017). However, very few studies have been conducted on cortical morphogenesis during encystment (Matsusaka *et al.*, 1989, Gu and Ni, 1995; Foissner *et al.*, 2007; Benčat´ova *et al.*, 2016). Ultrastructural studies and molecular genetics during encystment may provide a better understanding of the taxonomy and phylogeny of ciliates (Matsusaka,1977; Berger, 2006; Li *et al*., 2017; Chi *et al.,* 2020; Pan *et al*., 2021; Zhang and Vd'ačný, 2022). The ability to form cysts under unfavourable environmental conditions might have played a significant role in the long evoulutionay history of ciliates (Verni and Rosati, 2011).

The present study was conducted on the hypotrichous ciliate, *Pseudourostyla levis,* which was separated from other urostylids and given a generic status (Borror,1972), on the basis of morphological and morphogenetic characteristics. The ciliate *P.levis* is an elongate and dorsoventrally flattened cell, measuring 274.31± 13.4 µm in length and 80.02 ± 11.69 µm in width (mean ± S.D.,n =100). The cortical structures are typical of the genus *Pseudourostyla* and include buccal , frontal and somatic ciliature which are distinct ontogenetically and spatially (Berger, 2006). Buccal ciliature includes the adoral zone of membranelles (AZM) and undulating membranes (UMs). Frontal ciliature, also known as FVT complex, includes frontoventral cirri arranged in a bicorona, transverse cirri and an isolated malar cirrus. Somatic ciliature includes marinal rows (more than two rows on either side of FVT) and dorsal kineties. Nuclear apparatus consist of 23-59 macronuclei and 3-11 micronuclei.

In the Indian isolate of *Pseudourostyla levis*, a novel phenomenon of pseudoencystment was observed in which cells entered a state of dormancy after rounding up and secreted a soft mucoid capsule but did not form a cyst wall. The process of pseudoencystment was triggered by starvation in healthy cells.The cycle of pseudoencystment followed a programmed sequence of events, once it was initiated. Unlike true cysts, cells reverted to the vegetative state without waiting for favourable conditions to return. Alternately, on prolonged starvation, true cysts wers also formed which secreated cyst wall and required food or fresh culture medium for excystation. The pseudocysts were distinctly different from true cysts as they did not possess the cyst wall, and reverted back to active trophic state without providing food or fresh culture medium, and a synchronized induction of pseudocysts formation could be achieved by a brief spell of starvation. For this reason, this process was termed as pseudoencystment and an attempt was made to elucidate: (i) the parameters that induced pseudocyst formation, (ii) the temporal sequence of shape changes, (iii) corticomorphogenesis during pseudoencystment and excystment, (iv) experimental designed to illustrate the possible significance of this phenomenon in free-living ciliate *P. levis.*

MATERIALS AND METHODS

Culturing method

Water samples were collected from stagnant water bodies near the river Yamuna and water pools near Najafgarh area (28° 34' N, 76° 07' E), Delhi, India. Cultures were grown in petri dishes (180mm×30mm) and maintained in the BOD at 24±1°C, in the modified Pringsheim's medium. Cells were fed every 24 h with the green algae *Chlorogonium elongatum* (Ammermann *et al*., 1974). Axenic cultures of *Chlorogonium* were grown at 25°±1°C, under fluorescent lights set up with a photoperiod of 14 hrs.alternating with a dark period of 10 hrs. Under these conditions, optimum density of *Chlorogonium* was attained in three to four days after the initial inoculation. Algal cells were washed with Pringsheim's medium and used for feeding the ciliates. To maintain the ciliates in log phase, approximately, 550 *Chlorogonium/Pseudourostyla* were made available once in 24 hours. To avoid overcrowding, ciliates were divided in different petri dishes after three to four days.

Induction of pseudoencystment

Proliferating culture did not show pseudocyst formation. Preliminary observations revealed that the quantity of food and the nutritional status of the cells were the two most crucial factors in inducing pseudocyst formation. Accordingly, experiments were set up to establish the relationship between the onset of pseudoencystment and the quantity of food made available to the cells. For this purpose, a feeding protocol was standardized.

In the course of routine feeding (once in 24 hrs), ciliate cultures were provided with food material that was approximately 550 *Chlorogonium*: one *P. levis* cell. This was the optimum quantity of food, required to keep the ciliates in a healthy proliferative state. To assess the influence of food quantity on the process of pseudocyst formation, different dilutions of *Chlorogonium* were made (Table1). Number of *Chlorogonium* cells available per *Pseudourostyla* were from 550 to zero in different culture dishes. Each culture dish contained about 1000 *Pseudourostyla* cells/ml of the culture fluid. Pseudocysts formed within a period of 24 hours were scored.

To enumerate the number of *Chlorogonium* in a known

aliquot of culture medium, 0.1 ml of washed *Chlorogonium* cells were taken and counted using Neubauer,s chamber. The cells were counted in the five squres of central square meant for RBC counting. The number of *Chlorogonium* cells/ ml were calculated using the formula N×Dilution/ Area× depth, where N is the number of cells counted in five squres. Accordingly, desired dilutions were made for experimental purposes.

To study the corticomorphological changes, cells were stained with Párducz haematoxylin staining method (Párducz 1952, 1967). Cilates were fixed in the fixative mixture of 2% aqueous Osmium tetraoxide and saturated solution of aqueous mercuric chloride (6:1 respectively). After 15 minutes cells were washed with distilled water and treated with 1% iron alum (ferric ammonium sulphate) for 2 minutes. Again cells were washed with distilled water and stained with Heidenhein's haematoxylin for 20 minutes. After washing with distilled water, cells were dehydrated and mounted in Canada Balsam.

Analysis of the significance of pseudoencystment

To analyze the significance of the pseudoencystment process, one set of culture of *P. levis* was fed daily (without skipping even a single day) for 6 months and maintained under conditions in which no pseudocyst formation occurred (Experimental). The second set of culture, was not fed on weekends or holidays and cells were frequently undergoing pseudoencystment (Control). At varying intervals, cells were scored for their size, proliferative capacity and feeding profile in both experimental and control cultures. Size measurements were done in arbitrary units by an ocular micrometer (Leitz) and converted into metric units with the help of a stage micrometer. To study the feeding profile, number of *Chlorogonium* consumed / ciliate was calculated at an interval of seven days. To study the change, if any, in the proliferative capacity, the cell cycle (Generation time) was compared in the experimental with the controls.

RESULTS

Induction of encystment

Starvation triggered the process of pseudocyst formation in *Pseudourostyla levis.* Pseudoencystment was observed when the feeding was skipped in cultures with cells at the end of the log phase. After approximately 18 hrs (from the time when routine feeding was omitted), more than 40% of cells were noticed in stage 1 of pseudoencystment. The total period from the point of induction to the formation of a pseudocyst took about 24-26 hrs.

Effect of food availability on the formation of pseudocyst in *P.levis* was clearly indicated by the results obtained, when different dilutions of food were provided to an equal number of ciliates, maintained in separate culture dishes.Number of *Chlorogonium* available per ciliate was serially reduced from 550 to zero in parallel culture dishes. Data in Table 1 clearly showed that percentage of pseudocyst formed increased with the increase in food dilution. When the number of *Chlorogonium* / ciliate was reduced from 550 to 350, only 0.5% pseudocysts were obtained. With further diution of 200 *Chlorogonium/* ciliate, 13.6% pseudocysts were formed and complete withdrawal of food yielded a maximum of 42.9% pseudocysts. The result clearly indicated that the percentage of pseudocysts formed was directly proportional to the availability of the *Chlorogonium/ Pseudourostyla* cell.

Stages of pseudoencystment

To induce pseudocysts formation, routine feeding was skipped and it was counted as zero hour. The ciliates at this stage were elongate and dorsoventrally flat cells (Fig.1). However, with prolonged starvation morphologically identifiable cells were observed. On the basis of shape changes and cell size, chronological order of different stages of pseudoencystment and excystment, is presented in Table 2. Different stages of pseudoencystment and excystment were:

Stage 1: Cells in stage 1 were identifiable about 18 hours from the time of induction (time when the routine feeding was skipped). Stage 1 cells were slightly shorter in length as compared to vegetative cells and were spindle-shaped and had a dense cytoplasm (Fig. 2). Cells remained in this stage for approximately 4 hrs (Fig. 2). Cells were distinguished as they were slightly shorter in length, spindle-shaped and had a dense cytoplasm.

Stage 2: Cells became smaller and broader with blunt ends. A further increase in the cytoplasmic density was observed. This stage persisted for about 2 hrs (Fig. 3).

Stage 3: Cells gradually acquired a spheroid shape and at this stage they were referred to as young pseudocysts (Fig. 4). The cells remained in this stage for about 8 hrs. and then transformed into a mature pseudocyst or stage 4.

Stage 4: Cells at this stage were designated as mature pseudocysts (Fig. 5). The cells persisted in this stage for about 4 hrs and then started showing signs of excystment. Thus, cells remained for about 12 hrs in the rounded dormant state and did not feed during the entire process of pseudoencystment.

Corticomorphological changes during pseudoencystment

The ciliature of a vegetative cell (stage zero) includes buccal ciliature (AZM and UMs), FVT, right and left marginal cirral rows (Fig.6).The adoral zone of membranelles (AZM) extends up to 1/3-1/4 of the body length and consists of 84-113 membranelles. On the

right side of AZM, two undulating membranes (UMs) are present, the outer paroral and the inner endoral membrane. Frontal ciliature (FVT complex) consists of two rows of frontoventral cirri, 6-10 transverse cirri and an isolated malar cirrus. There are five rows of right and four rows of left marginal rows on either side of FVT complex. Dorsal surface is covered with seven rows of dorsal kineties.

During the transformation of vegetative cell to a pseudocyst (Figs.7-9), the relative positioning of cirri on the ventral surface, and dorsal kineties remained unaltered except that they were so adjusted to get accommodated in a smaller spheroid structure. Once a pseudocyst was formed, it remained in this rounded, dormant state for approximately 12 hours, during which there was substantial resorption of the AZM and the UMs. Pseudoencysted cells showed an average of 15 membranelles as compared to an average of 96 membranelles in vegetative cells.

Excystment

Pseudocysts stayed in the rounded, dormant state for about 12 hrs. afterwhich they reverted to vegetative state without reference to any change in the environmental conditions.The excysted cell was somewhat spindle-shaped.The shapes'changes during excystment were in a reverse order as compared to that during the pseudoencystment process.The excysted cell was smaller than a proliferating vegetative cell and resumed feeding after 6-8 hrs from when excystment started.

However, true cysts did not excyst unless fresh culture medium and *Chlorogonium* was provided. After the addition of food, cells started showing rotation inside the cyst wall within 4-5 hrs of induction and finally cyst wall burst to release the cell. Newly excysted cells were spheroids and started feeding within 6-7 hrs. Gradually they changed their shape and size and transformed into vegetative cells.

Analysis of significance of pseudoencystment

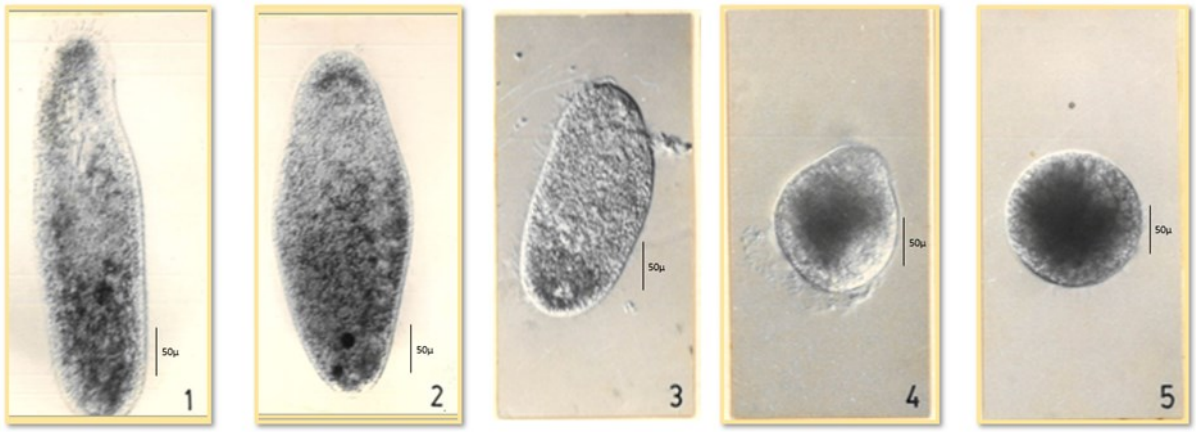
A comparison of cell size, generation time, and food consumption of controls and experimental ciliated did not reveal any significant change. Average generation time was the same in both control and experimental cells, i.e., 20.5±0.5 hrs. Average cell size observed in controls and experimental cells was 268.5 µm ×82.2 µm and 278.5µm µm × 80.5 µm, respectively. Cells from both cultures depicted similar efficiency in utilization of the food material.The pseudoencysted cells reverted back to the active trophic state after 43 hours (from the time of induction to the formation of an excysted vegetative cell), without food. Thus, the process appeared to be a programmed event. From this experiment, it was

Table 1. Effect of food availability on the formation of pseudocyst in *P. levis* The number of ciliates per ml. of the culture fluid was about 1000 cells

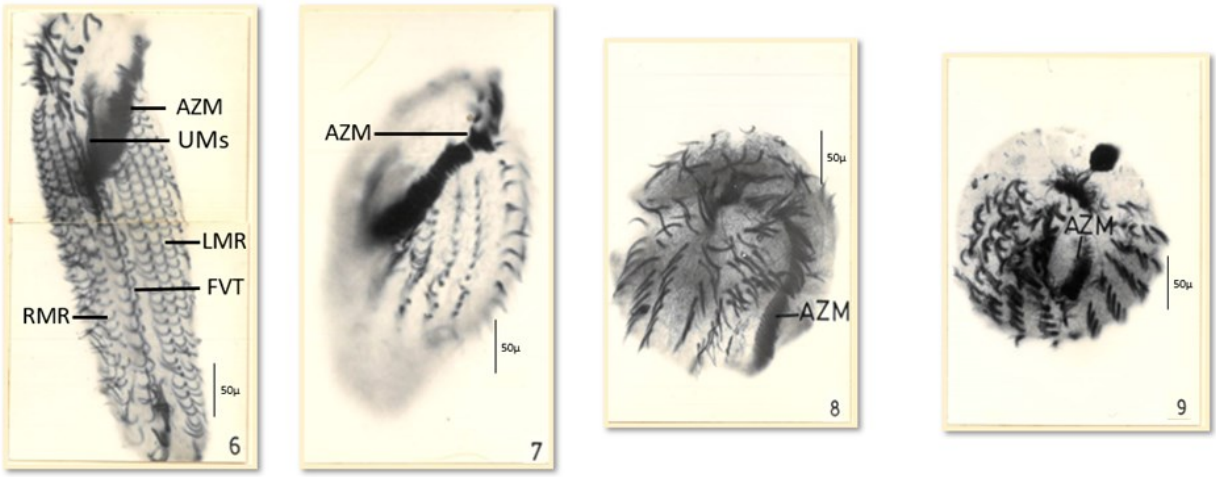
Number of *Chlorogonium* available/ *Pseudourostyla*	**Percentage of pseudocyst induction within 24**	**Remarks**
Approximately 550	0	Optimal food quantity and cells were in different stages of division
Approximately 500	0	
Approximately 350	0.5	
Approximately 200	13.6	With a decrease in the number of *Chlorogonium* cells available per *Pseudourostyla* cell, percentage of Pseudocyst formation increases.
Approximately 150	12.6	
Approximately 100	33.9	
0	42.9	

Table 2. Time Schedule of various stages of pseudoencystment and excystment from the point of induction of pseudocyst formation.

Time interval from the point of induction of pseudoencystment	**Stages observed**	**Cell size (µm)**
0 hr.	Vegetative cell	252.6-290.6× 64.3-115.9
18 hrs.	Stage 1	225-240 × 80-110
22hrs.	Stage 2	175-185 × 95-105
26 hrs.	Stage 3	110-115 in diameter
34 hrs.	Pseudocyst	-do-
38 hrs.	Early excysting cell	175-180 × 90-103
42 hrs.	Excysted cell	165- 172 × 80-85



Figs.1-5. *Photomicrographs of live cells of Pseudourostyla levis under Nomarski Phase Contrast microscope revealing morhological changes during pseudoencystment. × 450. Fig.1: Vegetative cell; Fig.2: Stage 1; Fig.3: Stage 2; Fig.4: Early stage 3; Fig.5: Stage 4 (pseudocyst).*



Figs. 6-9. *Photomicrographs of Pseudourostyla levis revealing cortical changes during pseudoencystment (Iron hematoxylin staining). × 450. Fig.6: Vegetative cell showing ciliary structures on the ventral surface: buccal ciliature includes adoral zone of membranelles (AZM) and undulating membranes (Ums), frontoventral complex (FVT), right marginal cirral rows (RMR) and left marginal cirral rows (LMR). Fig.7: A cell at late stage 1, showing resorption of Ums and resorbing membranelles of AZM. Fig.8: A cell at stage 3, showing all the ventral cirral rows are intact. Fig.9: A cell at stage 4, showing a substantial resorption of AZM.*

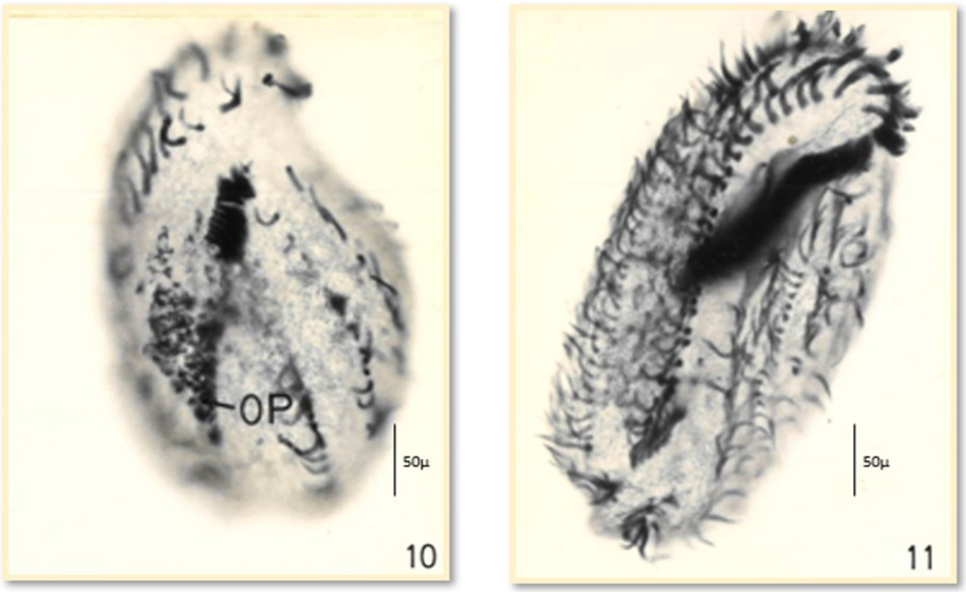
inferred that the process of pseudoencystment was not a rejuvenating process and appeared to be a short-term survival strategy evolved by the ciliate to overcome the period of starvation.

DISCUSSION

In the present study on *P. levis* a unique phenomenon of pseudoencystment with certain peculiarities was observed. Pseudocysts did not possess cyst wall yet they were dormant. The state of dormancy lasted only for a limited period of about 12 hours and appeared to be a programmed event. Reversion to the vegetative state was not dependent upon favourable conditions.

In *Urostyla grandis,*Pigon and Edström (1959) and Pigon (1960) reported the formation of round cells as a transitory stage during encystment. Later, the round cells formed cyst wall, had a tendency to stick to each other, retained ventral ciliature but completely resorbed the buccal ciliature.

True encystment has been described in *Pseudourostyla criststa* (Grim and Manganaro,1985 and Pan *et al*., 2019) but no intervening stages comparable to pseudocysts have been mentioned. In *Pseudourostyla levis*, Takahashi (1973) has described that during temporary conjugation, exconjugants undergo encystment and unlike true cysts of the same species, they lack cyst wall but show nuclear reorganization, which does not occur during true encystment. In the present study on pseudocysts, nuclear reorganization was not observed. In *Onychodromus* (Jareño, 1977), during conjugation, a process called joint encystment occurs which involves fusion of the cytoplasm of pairs, but no macronuclear reorganization ensues. Such pairs form cysts without cyst wall and excystment results in the formation of doublet cells. No such event of pairing or doublet for-



Figs.10-11. *Photomicrographs of Pseudourostyla levis revealing cortical changes on the ventral surface during excystment (Iron-hematoxylin staining). × 450. Fig.10: An excysting cell showing oral primordium (OP). Fig.11: An excysted vegetative cell, showing complete AZM and UMs.*

mation was observed in the present study. Hence the process of pseudoencystment differs from the reported encystation in other ciliates and is a unique phenomenon. Therefore, in the absence of any direct evidence revealing the significance of this process, a comparison of certain aspects is mentioned below so as to assess the proper significance of this phenomenon.

Induction of pseudoencystment

Food was the crucial factor in inducing pseudoencystment.The pseudocyst remained dormant for about 12 hrs and then reverted to the vegetative trophic state. The entire process, from the point of induction to the formation of an excysted vegetative cell, took about 43 hrs.Thus, the process of pseudoencystment appeared to be a short-term survival strategy evolved by the ciliate to survive periods marked by the anticipated deficiency of food. Protective cysts reported in other ciliates manifest a long-term survival strategy and wait till the adverse environmental conditions are over.

Absence of food evokes multifarious responses in different ciliates. These responses include formation of true cysts (Beers, 1927; Manwell, 1928; Beers, 1930; Corliss and Esser, 1974; Gutierrez *et al.,* 2001; Gao *et al.*,2015; Li *et al.,*2017; Matsuoka *et al.,*2021). In *Onychodromus quadricornutus*, starvation induced formation of cannibal giants (Wicklow ,1988).

Shape changes

Shape changes must involve enormous reorganization of the cytoskeletal elements in order to acquire a spherical configuration from a dorso-ventrally flattened, elongated structure. In the protective cysts of *Histriculus muscorum* (Nakamura and Matsusaka, 1985), most of the microtubular network of cells is completely depolymerized into a soluble tubulin pool. In *Euplotes encysticus,* enhanced levels of $\propto -tubulin$ (Chen *et al*., 2018) and in *Colpoda cucullus,* both $\propto$ and β $tubulin$ (Sogame *et al*., 2013), have been reported. Upregulation of these proteins indicate their involvement in the cytoskeletal reorganization during encystment. In *Pseudourostyla cristata,* Gao *et al*. (2015) identified encystment related proteins and suggested their possible role in stress tolerance and, subsequently, cyst formation. Pan *et al.* (2021) have compared the Inc RNAs profile and coexpressed RNAs in dormant cysts and vegetative cells of *P.cristata* and suggested their contribution to the process of encystment.

Cortico-morphological changes during pseudoencystment

During the transformation of a young pseudocyst to a mature pseudocyst, substantial resorption of AZM and UMs occured, whereas all the ventral cirral rows remained intact. Number of dorsal kineties also remained unchanged in a pseudocyst.

On the basis of the degree of resorption of ciliature, Matsusaka *et al*. (1989), classified the resting cysts of hypotrichs into three categories: (1) non-kinetosome resorbing (NKR) cysts of euplotids, containing intact cytoplasmic tubules, basal bodies and ciliary shafts, (2) partial kinetosome resorbing cysts of urostylids containing basal bodies but no ciliary shafts, and (3) kinetosome resorbing cysts of oxytrichids containing no microtubular organelles, except for *Paraurostyla weissei*, containing cortical microtubules.

In the true cysts of *Pseudourostyla cristata* (Grim and Manganaro,1985) cirri remains intact beneath the cyst

wall, but the AZM is completely resorbed. However, very few studies have been conducted on the mechanism of ciliature dedifferentiation and redifferentiation (Grimes,1973; Walker and Maugel,1980; Rios *et al*.,1985; Berger,2006; Li *et al*., 2017).

The present study on *P. levis* indicated that pseudocysts were unique and differed from the described types of cysts of hypotrichs. In pseudocysts, the partly resorbed AZM and UMs persisted and thus differed from both *Euplotes* type, where no resorption occurred and from *Urostyla* type, where complete resorption occurred.

During excystment of pseudocysts to active trophic state, a primordium equivalent to buccal primordium was formed and a complete AZM was reorganized in 3-4 hours. However, the AZM of the excysted cell had fewer membranelles than a vegetative cell. Other detailed stages, showing the reorganization process during excystment, could not be traced. Corticomorphogenesis during encystmsnt and excystment has been reported in few ciliates but the fate of ventral and dorsal ciliature is not described in detail (Gu and Xu 1995; Gao *et al.,* 2015; Benčaťová and Tirjaková 2017,2018; Jiang *et al.,* 2019; Jung *et al.,* 2021; Zhu *et al.,*2021; Li *et al.,*2022). Kamra and Sapra (1991) reported that during excysment, *Coniculostomum monilata,* required at least three division cycles for the complete regeneration of the ciliature.

Conclusion

Ciliates coexisting in habitat have evolved different strategies to survive. Encystment is a stress-induced phenomenon and an important bionomic strategy evolved by ciliates to overcome environmental challenges. The ability to encyst during the period of unfavourable environmental conditions might have played a significant role in the long evolutionary history of ciliates. The present study on *Pseudourostyla levis,* reveals that pseudoencystment is a short-term survival strategy adopted by the ciliate to survive the periods of starvation.The process of pseudoencystment and excystment in *P.levis* is a reversible cell differentiation and redifferentiation process and may provide significant information for understanding the unresolved aspects of phylogeny of ciliates. Molecular analysis of gene expression during pseudoencystment, is required to be done in *P. levis* to study the downregulated and upregulated proteins to understand their role in the process of dedifferentiation and redifferentiation.

ACKNOWLEDGEMENTS

I am thankful to Prof. G.R. Sapra, University of Delhi, who has been my mentor and helped me throughout the present work. I acknowledge Dyal Singh College, University of Delhi, for its continuous support, encouragement and motivation throughout the duration and preparation of this paper.

Conflict of interest

The authors declare that they have no conflict of interest.

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