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Encystace a životní cyklus volně žijících améb rodu *Acanthamoeba* spp.

Encystation and life cycle of free living amoebae of the genus *Acanthamoeba* spp.

Ph.D. Thesis

Supervisor: RNDr. Eva Nohýnková, Ph.D.

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Abstrakt

Améby rodu *Acanthamoeba* jsou celosvětově rozšířená, volně žijící, fakultativně patogenní jednobuněčná eukaryota. Jejich nebezpečnost pro člověka spočívá ve schopnosti pronikat do organismu, odolat obranným mechanismům, množit se, poškozovat napadené tkáně a tím vyvolat onemocnění, proti kterým chybí kauzální léčba a která nejčastěji postihují oko a centrální nervovou soustavu (CNS). Jedním z hlavních důvodů často neúspěšné terapie je schopnost akantaméb vytvářet v postižených tkáních cysty, vysoce rezistentní klidová stádia. Kromě cyst, které akantaméby tvoří jak v přírodě, tak v infikovaných tkáních pod vlivem dlouhodobého stresu, se tyto organismy vlivem akutního ohrožení rychle transformují v méně odolná klidová stádia, pseudocysty. Předkládaná práce se zaměřuje na dosud neznámé aspekty odolnosti obou rezistentních stádií akantaméb, cyst a pseudocyst, a současně si klade za cíl popsat další charakteristiky odlišující cysty a pseudocysty a procesy spojené s jejich tvorbou a rezistencí.

Jedním ze studovaných aspektů odolnosti klidových stádií akantaméb byla přítomnost cukerného alkoholu manitolu a neredukujícího cukru trehalózy, sacharidů, které se v buňkách mnoha organismů účastní obranných reakcí vůči abiotickému stresu. Ačkoli v genomu *A. castellanii* jsou enzymy pro syntézu obou cukrů popsány, v buňkách akantaméb jsme prokázali pouze přítomnost disacharidu trehalózy a to jak v klidových stádiích, cystách a pseudocystách, tak v aktivně se množících trofozoitech. Manitol se nám nepodařilo prokázat u žádné ze studovaných životních forem. Podrobnější analýza sekvencí genů kódujících enzymy podílející se na syntéze trehalózy, trehalóza fosfát syntázy (TPS), trehalóza fosfát fosfatázy (TPP) a trehalóza syntázy (TS), odhalila, že v genomu se nachází enzymy dvou syntetických drah, z nichž jedna je prokaryontního původu. qRT-PCR pak prokázala rozdíly v míře exprese těchto genů v závislosti na dané životní formě, cysty vs. pseudocysty. V průběhu tvorby pseudocyst se na syntéze trehalózy podílí geny obou syntetických drah a v mnohem větší míře, než je tomu v průběhu encystace. K nárůstu množství trehalózy však dochází v průběhu obou ochranných reakcí. Trehalóza přetrvává, i když v menší míře, i v buňkách zralých cyst a pseudocyst, jak bylo doloženo pomocí hmotnostní spektrometrie.

Studovali jsme také vztah mezi encystací resp. tvorbou pseudocyst a buněčným cyklem resp. množstvím DNA. Pomocí průtokové cytometrie jsme prokázali, že u akantaméb lze jasně rozlišit dvě populace buněk s odlišným množstvím DNA, populaci v G1 fázi buněčného cyklu a populaci s již nově nasyntetizovanou DNA v G2 fázi. Zjistili jsme, že zatímco encystaci vždy předchází syntéza DNA, probíhá tedy z G2 fáze buněčného cyklu,

tvorba pseudocyst je na pozici v buněčném cyklu nezávislá a může k ní dojít kdykoli během buněčného cyklu. Co je však pro obě rezistentní stádia totožné, je obsah DNA u zralých cyst a pseudocyst. Obě rezistentní stádia přečkávají dobu nepříznivých vnějších podmínek s G2 fázovým, obsahem DNA. Popsali jsme také účinek inhibitorů afidikolinu a hydroxyurey na růst a buněčný cyklus akantaméb. Zjistili jsme, že ani jedna ze studovaných látek nesynchronizuje akantaméby a nezastavuje je v množení na rozhraní G1/S fáze, jak je to popsáno u jiných protozoií a savčích buněk. Současně jsme pozorovali koncentračně závislý vliv hydroxyurey na míru množení trofozoitů akantaméb. Klasifikace a následná fylogenetická analýza hlavních regulátorů buněčného cyklu odhalila přítomnost celkem 14 genů 9 typů cyklinů a 6 genů 3 typů cyklin-dependentních kináz.

Abstract

Amoebae of the genus *Acanthamoeba* spp. are free-living unicellular organisms found in disparate ecosystems all over the world. Due to their ability to invade human body, evade its defensive mechanisms and cause extensive tissue damage, *Acanthamoeba* infection can lead to serious, if rare, diseases, affecting most commonly the eye and the central nervous system. Specific therapy for *Acanthamoeba* infections is not available.

A major reason for therapeutic failure in ameobiasis is the ability of the protist to differentiate into resistant stages. These are *cysts*, known to be formed under prolonged unfavorable conditions, both in the environment and the infected tissues, and the *pseudocysts*, less durable but rapidly formed under acute stress. The present thesis focuses on as yet unexplored mechanisms of resistance of cysts and pseudocysts. Moreover, further characteristics distinguishing cysts and pseudocysts as well as the processes involved in their formation are investigated.

One of the issues addressed is a presence of protective carbohydrate compounds mannitol and trehalose that participate in defensive reactions against abiotic stress in many organisms. Although putative genes for enzymes of the trehalose and mannitol synthetic pathways are present in the genome of *Acanthamoeba*, only one of the two compounds, disaccharide trehalose, was found. Trehalose was identified not only in cysts and pseudocysts but also in growing trophozoites. In contrast, none of the life stages were shown to contain mannitol. Detailed analysis of the sequences of the enzymes of the trehalose synthetic pathways, trehalose phosphate synthase (TPS), trehalose phosphate phosphatase (TPP) and trehalose synthase (TS), revealed that the genome contains enzymes belonging to two distinct enzymatic pathways, one being of a prokaryotic origin. Quantitative RT-PCR demonstrated a significant difference in expression profiles of the synthetic pathway genes in cyst and pseudocyst. Genes of both synthetic pathways are involved in trehalose synthesis during pseudocyst formation and at higher level than during encystation. Amounts of trehalose are nevertheless increased during both stress defense reactions. The presence of trehalose in mature cysts and pseudocysts was also demonstrated using mass spectrometry.

Furthermore, the relationship between encystation and pseudocyst formation and the progress of the cell cycle was also studied. Phylogenetic analysis and classification of the main cell cycle regulators in the *Acanthamoeba* genome revealed presence of 14 genes of 9 types of cyclins and 6 genes of 3 types of cyclin-dependent kinases.

By using flow cytometry analysis we clearly distinguished cell populations with distinct DNA content, G1 cell cycle population and population with newly synthetized DNA,

G2 population. Our results strongly indicate that *A. castellanii* enters encystation from the G2 phase of the cell cycle. In contrast, initiation of differentiation into pseudocysts is independent of the progression of the cell cycle. Nevertheless, DNA content in mature cysts and pseudocysts is the same, both resistant stages survive harsh environmental conditions with G2 phase DNA content. We also described the effect of DNA synthesis inhibitors aphidicolin and hydroxyurea on *Acanthamoeba* growth and cell cycle. Our data revealed that neither of the studied compounds synchronized *Acanthamoeba* cell populations on the G1/S boundary, in contrast to what was described in other protists as well as mammalian cell lines. Along with this, we did observe concentration dependent impact of hydroxyurea on *Acanthamoeba* trophozoites growth rate.

List of publications

I. Bínová E, Klieščiková J, Ashford D, Thomas-Oates J, Nohýnková E (2012). Mannitol is not involved in protective reactions of *Acanthamoeba*. Molecular and Biochemical Parasitology 184:118-121

Impact factor: 2.158 (2018)

II. Bínová E, Bína D, Ashford DA, Thomas-Oates J, Nohýnková E (2017). Trehalose during two stress responses in *Acanthamoeba*: Differentiation between encystation and pseudocyst formation. Protist 168:649-662

Impact factor: 3.000 (2018)

III. Bínová E, Bína D, Nohýnková E (2020). DNA content in *Acanthamoeba* during two stress defense reactions: Encystation, pseudocyst formation and cell cycle. European Journal of Protistology (in press) doi: 10.1016/j.ejop.2020.125745.

Impact factor: 2.395 (2019)

Contents

1. Introduction

1.1. Protective/stress reactions in protists

1.1.1. General considerations

All types of organisms have the ability to respond to changes in environmental conditions. Such responses require a complex network of sensing and signal transductions leading to adaptations of intracellular processes such as cell growth, gene expression, metabolic activities and other features of the cell (Hohmannn 2002). Single-celled organisms, in particular, must contend with fluctuations in nutrients, pH, temperature, and external osmolarity, as well as exposure to UV irradiation and a range of potentially toxic environmental compounds. Appropriate responses to these environmental stresses must be induced for cell survival and proliferation (Chen 2003).

From a biological point of view, we can distinguish two types of stresses; abiotic and biotic ones. Abiotic stress includes all exogenous physicochemical environmental factors such as temperature, pH, inorganic (metals, metalloids, metallic nanoparticles) and organic (xenobiotic) compounds, starvation, drought, etc.), biotic stress involves the presence of whole cells or organisms acting as the stress source, and the interaction between the living

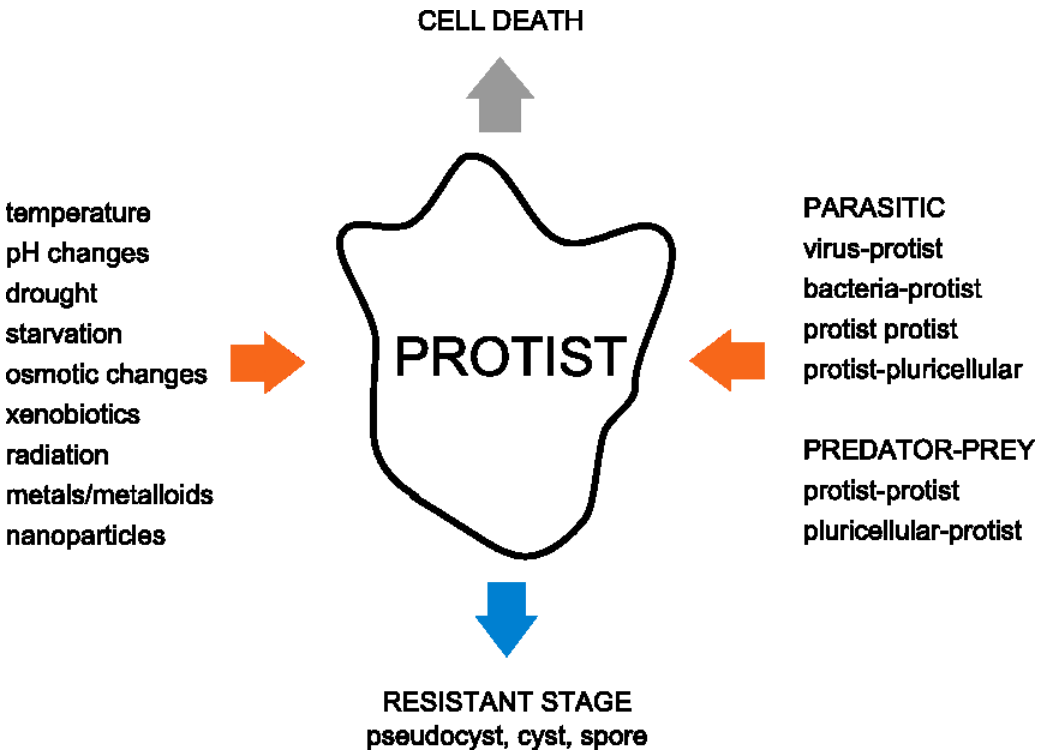

Fig. 1 Reaction of a protist to stressful environmental conditions, after Slaveykova et al. (2016)

stressor and the living receptor is the real cause of the stress (Fig. 1) (Slaveykova et al. 2016).

Cell mechanisms to respond to environmental changes are universal, so, in general, they are present in all living beings, including protists. The cell response can be specific to only one stressor or general (common response) to several different stressors (Swiecilo 2016). Depending on the nature of stressor, cell adaptive responses may consist in a readjustment of metabolism or induction of new gene expression (Ruis 1997). Moreover, the new gene expression can lead to cell differentiation process resulting in a resistant cellular stage. All these cell alterations are focused to maintain cell survival under the stress conditions and the failure to employ the protective mechanisms results in cell death (Fig. 1). It should be noted that convergent points exist among various types of stresses both in the signal transduction networks and overlap of gene clusters participating (Fujita et al. 2006). In particular, generation of reactive oxygen species (ROS) and MAP-kinase cascades deserve mention (Apel and Hirt 2004, Nakagami et al. 2005; Swiecilo 2016).

1.1.2 Protective mechanisms and molecules

The biological aim of the stress response is to protect the cell components against the potentially dangerous effects of stress factors. The goal of the process is reduction of synthesis of most proteins produced in the cell in normal physiological conditions and induction of synthesis of a specific group of proteins known as stress proteins. Stress proteins include heat shock proteins (Hsp), enzymes involved in cellular redox reactions and antioxidant defense, as well as enzymes active in the metabolism of carbohydrates and fatty acids, DNA repair, cell wall modification, metabolite transport, the functioning of mitochondria and vacuoles, autophagic processes, and intracellular signal transduction (Gasch et al. 2000).

Although there exist universal mechanisms of cell response to environmental cues, particular realizations of the stress response systems reflect the life strategy and evolutionary history of individual species. In a group defined as broadly as protists, a comprehensive treatment of this issue exceeds the scope of this work and reader is referred to recent literature on important model groups: in free living protists, several types of stress responses to different environmental factors have been studied in ciliates (Dondero et al. 2004; Ferro et al. 2015; Gutierrez et al. 2001, 2008, 2011; Gutierrez and Martin-Gonzalez, 2002; Kim et al. 2014; Rico et al. 2009; Zou et al. 2013) and also in amoebozoans *Dictyostelium discoideum* (King et al. 2011; Ott et al. 2000; Sun et al. 2003; Thewes et al. 2014; West et al. 2009) and *A.*

castellanii (Goncalves et al. 2018; Klieščikova et al. 2011a,b; Motavalli et al. 2018; Woyda-Ploszczyca et al. 2011).

In parasitic protists, major interest is in the investigation of the oxidative stress response because the generation of reactive oxygen and nitrogen intermediates by macrophages, neutrophils and other phagocytic cells constitutes a key part of the immune response of the host organism (Bogdan et al. 2000). One of the more intensely studied organisms in this respect are: *Entamoeba histolytica* (Cabeza et al. 2015; Choi et al. 2005; Dam et al. 2019; Hughes et al. 2003; Weber et al. 2006), *Giardia intestinalis* (Di Matteo et al. 2008; Lindley et al. 1988; Raj and Ganguly 2019; *Trichomonas vaginalis* (Coombs et al. 2004; Nývltová et al. 2016; Singh et al. 2018; Smutná et al. 2009) and *Plasmodium falciparum* (Day et al. 2019; Fairfield et al. 1988; Sussman et al. 2017).

With respect to the amphizoic amoebozoa that are the focus of the thesis, of particular interest is cellular differentiation into stress-resistant stages and a synthesis of protective compounds. Here, we present a brief overview of the topic, a more detailed treatment can be found in respective following chapters.

Cellular differentiation into stress-resistant stages, spores, pseudocysts or cysts represents an important mechanism of dealing with harsh environmental conditions. These stages enable the organisms to survive until the conditions improve, maintaining viability over extended time period, even several decades (Mazur et al. 1995; Sriram et al. 2008). Resting stages formed to survive unfavorable environmental conditions, as opposed to reproduction or dissemination, are well documented in a number of major groups of free living protists, e.g. ciliates (Gutierrez et al. 1990), diatoms (Smetacek 1985), dinoflagellates (Kremp et al. 2009), excavates (such as amoeboflagellate *Naegleria*, (Marciano-Cabral, 1988), and Euglenozoa, (Hindák et al. 2000) and of course amoebozoa, where the ability to encysts is widespread (Schaap and Schilde 2018). For a recent review summarizing the topic of encystation across the whole eukaryote kingdom see Schaap and Schilde (2018).

A major role in the resistance of these stages is played by the structure and composition of their surface layers. On the cyst surface of free living amoebae such as *Acanthamoeba* spp., *Balamuthia mandrillaris*, *Hartmannella* spp. or *Naegleria* spp. there are multi-layered cyst walls composed of sugars and proteins. According to majority of literature, one of a typical cyst wall component present in *Acanthamoeba* spp., and also in *B. mandrillaris* is the polysaccharide cellulose (Tomlinson et al. 1962; Siddiqui et al. 2009). However, even such a seemingly straightforward issue of the chemical composition of the cell

wall remains possibly contentious: the absence of cellulose in the cell wall of *Balamuthia* cysts was reported by Klieščiková (2013).

Chitin, another insoluble polysaccharide with comparable structure found mainly in cell walls of fungi and insects' bodies was detected in the cyst wall of *Naegleria* spp. (Linder et al. 2002) and also in *Entamoeba invadens* (Arroyo-Begovich et al. 1980).

Soluble sugars, such as mono- and disaccharides as well as raffinose family oligosaccharides, can function as osmolytes to maintain cell turgor, to protect membranes and proteins, and to act as radical scavengers upon abiotic stress conditions. They are thought to play a crucial role in acclimation to osmotic stress conditions such as drought, cold, and salt stress (Krasensky and Jonak 2012). Also, these type of sugars were detected in the resistant stages, *Giardia* (Jarroll et al. 1989), *Acanthamoeba* (Dudley et al. 2009) and *Balamuthia* (Siddiqui et al. 2009) cysts and in spores of the social amoebae *Dictyostelium discoideum* (Emyanitoff and Wright 1979; Jackson et al. 1982).

1.2. *Acanthamoeba* as a model organism

1.2.1. *Acanthamoeba* as a free - living amoeba

1.2.1.1. Taxonomy

Acanthamoeba spp. are early branching eukaryotic organisms belonging into protozoan phylum Amoebozoa. Amoebozoa is a monophyletic group (Cavalier-Smith et al., 2015; Brown et al., 2013) sister to Obazoa, the lineage that contains the animals and Fungi, as well as their protistan relatives (Tice et al., 2016; Kang et al., 2017). It evolved from their last common ancestor around 1.2 billion years ago (Eme et al. 2014). Amoebozoa is a very diverse group, both morphologically and ecologically: it contains naked amoebae species, testate amoebae (i.e., amoebae with a shell), amoeboid flagellates as well as those with cysts, spore or sexual states in their life cycle (Brown et al., 2007; Lahr et al., 2011; Tekle et al., 2017; Shadwick et al., 2009; 2016; Kudryavtsev et al. 2014). These aspects, together with the lack of molecular data of many of the species, are the main reason why evolutionary relationships within the Amoebozoan group are currently not well understood.

The recent work by Cavalier-Smith et al. (2015) classifies Amoebozoa into two basal monophyletic subphyla Lobosa and Conosa with six subgroups: Cutosea, Discosea, Tubulinea, Myetozoa, Variosea and Archamoebea (Fig. 1A). A different phylogeny was suggested by Kang et al. (2017) who divide the group Amoebozoa to two major clades,

Tevosa and Discosea, where the Tevosa contains the Tubulinea and Evosea groups based on a much larger sample in terms of number of species (61 amoebozoan taxa) and almost two times larger number of genes (325 genes) analyzed (Fig. 2B)

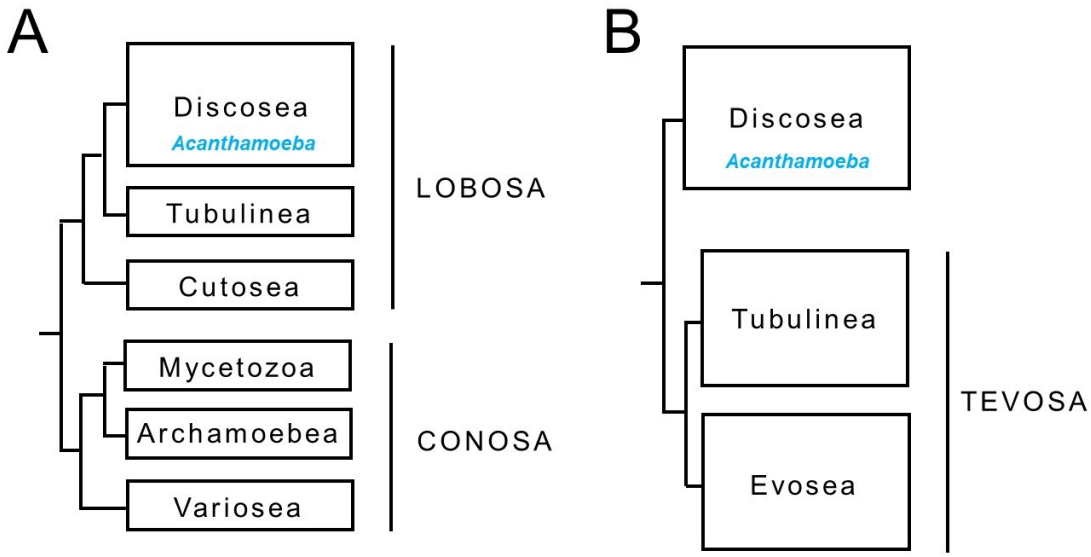

Fig. 2 Current opinions on Amoebozoa phylogeny. Simplified trees showing two possible phylogenetic reconstruction of Amoebozoa were adapted from Cavalier-Smith et al. (2016) (A) and Kang et al. (2017) (B). Both works consistently place *Acanthamoeba* within Discosea.

The phylogenetic position of the genus *Acanthamoeba* appears to be rather stable within the Amoebozoa group. All authors referenced above as well as Tice et al. (2016), place it into the Acanthamoebidae family as a part of Centramoebida and Discosea. The Acanthamoebidae is a well-established monophyletic group composed of five genera (*Luapeleamoeba*, *ProtAcanthamoeba*, *Acanthamoeba*, *Dracoamoeba* and *Vacuolamoeba*) represented by amoebae with a broad range of morphologies. It includes species with complex life cycles including formation of dormant stages, cysts and spores (Tice et al., 2016).

1.2.1.2 The life cycle

Two distinct stages are observed in the *Acanthamoeba* life cycle, an active trophic amoeba and a dormant cyst. A unique case appears to be *Acanthamoeba pyriformis* in which facultative sporocarpic fruiting was described recently (Tice et al., 2016).

Under favourable environmental conditions (food supply, neutral pH, appropriate temperature i.e., 30°C) *Acanthamoeba* exists as a motile and metabolically active trophozoite stage. It feeds on organic particles as well as microorganisms and multiplies by binary fission

with generation time of 8-24 hours (Khan 2009). *Acanthamoeba* traction is relatively fast with a rate approximately 0.8 μm per second. The movement involves the formation of characteristic hyaline pseudopodium. On the trophozoite surface there are also spine-like structures, acanthopodia, which surprisingly do not play role in *Acanthamoeba* movement on a flat surface, but it is supposed that within the soil acanthopodia might be able to exert traction by bracing the cell to the walls of interstices of the soil (Preston and King 1984). Like other eukaryotic cells, *Acanthamoeba* trophozoite contains membrane systems including rough and smooth endoplasmic reticulum, Golgi complex, free ribosomes, one prominent contractile vacuole, lysosomes, digestive vacuoles, glycogen-containing vacuoles, a large number of mitochondria and a nucleus. Contractile vacuole, whose function is to expel water, accompanied by tubular system (spongiome) enables the trophozoite to adapt to habitats with different osmolarity. The vacuole discharges periodically, fragmenting into a number of collapsed vesicles which subsequently refill (Bowers and Korn, 1968, 1973; Lasman, 1982; Gonzales-Robles et al., 2001).

Exposure of *Acanthamoeba* trophozoite to harsh environmental conditions (low or high pH, starvation, desiccation, cold, heat etc.) results in the process called encystation. At the beginning of encystation trophozoite loses its amoeboid appearance and becomes rounded. The trophozoite becomes gradually metabolically inactive or with just a very low metabolic activity and encloses itself within a resistant double-layered shell (Weisman 1976). The wall has been shown to contain polysaccharides cellulose (Tomlinson and Jones 1962, Garajová et al. 2019) and chitin (Magistrado-Coxen et al. 2019) in both layers, although localization of synthesis of polysaccharides to either plasmatic membrane or secretory vesicles (e.g., Magistrado-Coxen et al. 2019) remains an open issue (Stewart and Weisman 1974, Garajová et al. 2019) An outer cyst wall layer named ectocyst (also called exocyst by some authors) is formed first. It is 0.3 to 0.5 μm thick, wrinkled with folds and ripples and its major component are acid-insoluble proteins and lipids. The inner wall, endocyst, is usually polygonal, round or oval. Its major component is cellulose, which is also accompanied by proteins (Martínez and Visvesvara, 1997). As shown by a recent detailed analysis, the dominant cyst wall proteins can be classified into three families, one of these (labelled Jonah) is localized in the exocyst and is synthesized early during encystation, the other two groups (Luke and Leo) are specific for endocyst.

Cyst wall synthesis is usually accompanied by a decrease in cytoplasmic mass of approximately 80% through a gradual dehydration of the amoeba. Rather early, autolysosomes appear and remain in the cytoplasm throughout the whole encystment process.

There is no contractile vacuole in the cyst. Golgi apparatus is distributed into small aggregates. Rough endoplasmic reticulum, lipid droplets, glycogen particles and autolysosomes containing debris of mitochondria are found in the cyst through the process of encystation. (Bowers and Korn, 1969). There were described large aggregates of rod-shape elements similar to chromatoid bodies in *Acanthamoeba* mature cysts (Chavez-Munguia et al., 2013). As a cyst stage *Acanthamoeba* can survive for several years (Mazur et al., 1995; Köhsler et al., 2008; Sriram et al., 2008).

At the junction of the ecto and endocyst, the cyst possesses pores known as ostioles, which are possibly used to monitor environmental changes (Khan 2006). Under the favourable conditions *Acanthamoeba* emerge from the cyst leaving behind the outer shell by the process called excystation. The contractile vacuole becomes evident firstly in the pre-emerged stage. Then the stage moves through the ostiole to leave the shell behind. The emerging trophozoites have no acanthopodia. These appear upon completed excystation. The emerged trophozoite moves and reproduces leaving the empty cyst walls (without digestion) and the life cycle is completed (Chambers and Thompson, 1976; Dudley et al. 2008).

1.2.1.3. Distribution and Ecology

Acanthamoeba spp., as a free living amoeba, is an organism with a worldwide distribution. It was isolated from a wide range of environments. There are reports about *Acanthamoeba* findings in soil, mud, shoreline soils, pond and river water, hot spring resorts, fresh and salt water lakes, sea water, ocean sediments, beaches, water-air interface and even from the air (Kingston and Warhurst 1969; Kilvington and White 1994; Clarke and Niederkorn 2006). *Acanthamoeba* was also found in sub-Antarctica, McMurdo Sound-Dry Valley region in Antarctica and in two regions in the Arctic, Spitzbergen and Greenland (Brown et al., 1982; Bamforth et al., 2005; de Jonckheere 2006). The widespread distribution of *Acanthamoeba*, including extreme environments, signifies that this organism can adapt to and survive in very harsh environmental conditions. This ability is undoubtedly enabled also by its ability to create the resistant cyst form as mentioned above.

In addition to the wide range of natural habitats *Acanthamoeba* has been isolated from various human-made environments. It was found in air-conditioning units, ventilation, humidifiers, shower heads, kitchen sprayers, thermally-polluted factory discharges and cooling towers of power plants. Acanthamoebae have been recovered from hospitals, swimming pools and even from bottled mineral water, distilled water bottles and vegetables (Lyons and Kapur 1977; Gianinazzi et al. 2009; Siddiqui and Khan 2012).

Taken together, *Acanthamoeba* is a protozoan microorganism present in a broad range of habitats, including the human-made ones. So, it comes as no surprise that the humans themselves can occasionally serve as another niche of *Acanthamoeba* life. And not only humans. *Acanthamoeba* has been isolated also from various animals such as dogs, cows, pigs, rabbits, pigeons, sheep, reptiles, fish, turkeys, keel-billed toucan and horses (Taylor 1997; Van der Lugt and Van der Merwe 1990; Kinde et al. 2007).

One more aspect of *Acanthamoeba* life and ecology should not be omitted. Acanthamoebae, like other soil protists, play a major role in influencing the structure of microbial community by feeding on bacteria. They are the dominant bacterial consumers responsible for up to 60% of the total reduction in bacterial population (Sinclair et al., 1981). On the other hand, many bacteria species including the pathogenic ones such as *Legionella pneumophila, Listeria monocytogenes, Staphylococcus aureus, Mycobacteria tuberculosis, Pseudomonas aeruginosa, Rickettsia, Salmonella typhimurium* and others (Khan 2006; Guimaraes et al., 2016) can survive in cytosol of the *Acanthamoeba* trophozoites and even of the cysts (Kahane et al., 2001; Lambrecht et al., 2015). Relationships between *Acanthamoeba* and the hosted microorganisms are so far little understood. It is assumed that *Acanthamoeba* can serve as a "Trojan Horse" for many of them, to protect them from external conditions and transport them to "susceptible" environment, for example into the human body (Greub and Raould 2004).

1.2.2. Acanthamoeba as an amphizoic amoeba

The ability of acanthamoebae to live, in addition to the natural environment, a parasitic life style inside the body of some vertebrates, is placing them along with other amoebae, e.g., *B. mandrillaris*, *Naegleria fowleri* and *Sappinia pedata* in humans and other mammals, into the group of so called amphizoic amoebae. *Acanthamoeba*, after having invaded the human body, can cause severe diseases affecting eyes, brain and skin.

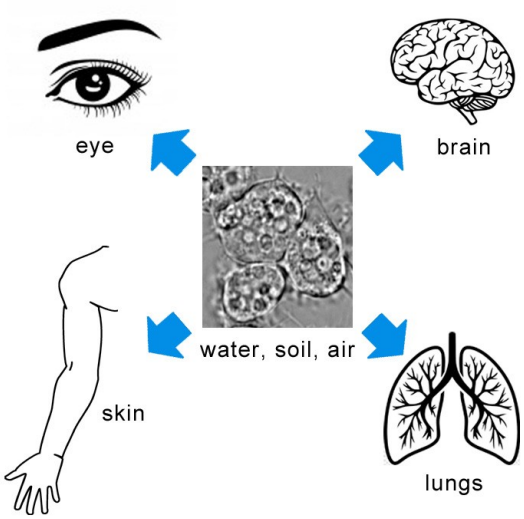

Fig. 3 Targets of *Acanthamoeba* infection.

1.2.2.1. *Acanthamoeba* keratitis

Acanthamoeba keratitis (AK) which is a chronic sight-threatening cornea infection is the most frequent disease caused by acanthamoebae. Since the first cases identified in Britain (Naginton et al. 1974) and USA (Jones et al. 1975) in seventies of the 20th century, thousands of cases of AK has been described from all over the world. Far the most cases of AK (85–88%) are connected with wearing contact lenses; of these, 88% cases are associated with wearing hydrogel lenses, and 12% with using rigid lenses (Dart et al. 2009). Seal (2003) reports that the annual probability of contracting AK for persons wearing contact lenses is 1:30 000. *Acanthamoeba* can cause keratitis also in people who do not wear contact lenses, most frequently after eye injury. Patients with AK suffer from pain with photophobia, ring-like stromal infiltrate, epithelial defect and lid oedema. In severe cases, the infection can terminate in loss of vision or even eye enucleation in the most severe cases (Marciano-Cabral and Cabral 2003; Clarke and Niederkorn 2006; Visvesvara et al., 2007). The treatment of amoebic infection of the cornea is extremely difficult and protracted (Yoder et al. 2012). It very often fails due to late or misdiagnosis, lack of causal effective treatments and existence of a highly resistant *Acanthamoeba* cyst stage causing chronicity of the disease (Dart et al. 2009).

1.2.2.2. *Acanthamoeba* encephalitis

Granulomatous Amoebic Encephalitis (GAE) is a rare, chronic, slowly progressive, focal central nervous system (CNS) infection. Although enhanced GAE susceptibility is associated with immune suppression and other debilitating conditions, a few cases of GAE have been recorded also in immunocompetent children and adults (Marciano-Cabral and Cabral, 2003). The first clearly identified human case of GAE occurred in the USA in 1972 (Jager and Stamm 1972). The first European case of GAE was reported in an AIDS patient from Italy in 1992 (Di Gregorio et al. 1992). Acanthamoebae invade the CNS via bloodstream either from the low respiratory tract after inbreathing contaminated air or from the contaminated skin. Whether acanthamoebae, similarly to *N. fowleri*, can directly invade the brain via destruction/phagocytosis of the olfactory epithelium as found experimentally is unknown (Khan 2009). Symptoms of GAE are non-specific corresponding to chronic CNS infection, i.e., headache, confusion, nausea, vomiting, fever, lethargy, stiff neck, focal neurologic deficits, or signs of increased intracranial pressure (Martinez and Visvesvara, 1997).

Pathological findings generally include severe haemorrhagic necrosis, fibrin thrombi, and inflammation. In addition, dissemination of amebae to other organs such as the liver, kidneys, trachea, and adrenals can occur in immunocompromised individuals (Murakawa et al., 1995).

1.2.2.3. *Acanthamoeba* cutaneous infections

Cutaneous infections caused by *Acanthamoeba* may represent a primary focus of infection or can be the result of hematogeneous dissemination from other sites such as the respiratory tract, sinuses, or the CNS (Friedland et al., 1992). They are most common in patients with AIDS (Casper et al., 1999), but have also been documented in patients undergoing immunosuppressive therapy or those suffering from immunological diseases (Slater et al., 1994; Oliva et al., 1999). The cutaneous form of the disease is characterized by the presence of large necrotizing plagues, hard erythematous nodules or skin ulcers (Bonilla et al., 1999; Levine et al., 2001). The reported mortality rate from cutaneous infection for individuals without CNS involvement is approximately 73%, while that from cutaneous infection accompanied by CNS disease is 100% (Torno et al., 2000).

1.3. *Acanthamoeba* genome

So far, 19 *Acanthamoeba* genotypes (T1 - T19), based on analysis of the 18S rRNA gene, have been described (cit). The *Acanthamoeba* genotypes do not necessarily correlate to 21 species established by morphology but allow appreciation of how diverse this genus is and can explain its worldwide distribution and its ability to colonize very different environments (Magnet et al., 2014). According to Maciver et al. (2013), T4 is the most common genotype both in the environment and as the causative agent of diseases. Genotypes T3 and T11, phylogenetically close to T4, are the other genotypes that have been associated with human infections. Genotypes T2, T5, T6, T10, T12, T15, and T18 were also described as pathogenic (Booton etal. 2005; Qvarnstrom et al. 2013; Stothard et al.1998; Walochnik et al. 2008).

Early studies estimated haploid *Acanthamoeba* genome sized 40-50 Mb (Bohnert and Hermann 1974; Jantzen et al. 1988). By the genome sequencing project, the size was determined to be 42.02 Mb (Clarke et al., 2013) with 14974 protein-coding genes identified. The *Acanthamoeba* genome appears to contain genes with a high number of introns (~6.7 exons per gene) and have 58% of GC content (Clarke et al., 2013; Karlyshev 2019). The analysis of Clarke et al. (2013) identified 450 genes, or 2.9% of the *Acanthamoeba* proteome, expected to have a prokaryotic origin. The existence of the lateral gene transfer (LGT) phenomenon between *Acanthemoeba* and bacteria have been recently corroborated also by

Wang and Wu (2017) and what's more, Chelkha et al. (2018) suggested LGT genetic exchanges among other *Acanthamoeba* endosymbionts, giant viruses. LGT is an all-encompassing term for the movement of DNA between diverse organisms. Originally, LGT was thought to occur primarily between closely related bacterial species. With the recent development of sequencing technologies, the number of identified LGTs from prokaryotes to eukaryotes has increased dramatically in the past 10 years (Karsten et al., 2017). LGT is considered a key process of genome evolution and several studies have indicated that phagotrophs manifest an increased rate of LGT compared to non-phagotrophic organisms (Keeling 2008). *Acanthamoeba* as a phagotrophic organism living in close relationship with bacteria and viruses in the external environment seems to be an ideal organism to participate in LGT process. According to Wang and Wu (2017), *Acanthamoeba* can serve a role of a "melting pot" for LGT among their endosymbiotic bacteria.

Researchers *Acanthamoeba* using data from *Acanthamoeba* genome database are faced with a question which of the numerous genome sequences (15 nuclear genome sequences and 16 mitochondrial DNA sequences in 2017) annotated for *Acanthameoba* to use. The first genome released for use by the community was that of the Neff strain **(**ATCC 30010) in 2013**.** This well annotated genome should have served as a template for comparison of other genome sequences. However, further studies (Crary 2012; Fuerst 2014, Corsaro 2020) revealed that this morphologically well characterised isolate is not only distant from the original type strain *A. castellanii* T4A, described by Castellani in 1930 but also clearly distinct from all other strains (subtypes) within T4, representing also a unique mitochondrial subtype (Ledee et al. 2003). In the light of the recent work, it thus appears that it is not adequate to consider Neff as a strain of *A. castellanii* and even a representative of the T4 genotype (Fuerst 2014). Consequently, choosing this isolate as a representative of the genus for genome sequencing has been rather unfortunate. In spite of this, there are numerous works in which *Acanthamoeba sp. Neff* has been used as a typical member of the pathogenic T4 genotype (Aqeel et al., 2012; Du et al., 2014; Jha et al., 2014; Moon et al., 2015), even though it is a non-pathogenic environmental isolate. The main problem with using this strain is that some genes for pathogenicity manifestation could be lacking and also some genes can be lost due to long term cultivation (from 1957) without natural environmental selection (Fuerst 2014).

Nowadays, although numerous genome sequences of *Acanthamoeba* spp. are accessible, many basic questions remain unclear or completely unanswered. There is still no conclusive evidence regarding number of chromosomes, ploidy level or the exact course of

the cell cycle. Looking for answers to some of these questions is in the focus of the present PhD Thesis.

1.4. *Acanthamoeba* cell protection - cyst and encystation vs. pseudocyst and pseudocyst formation.

Each organism has a range of environmental conditions in which it is able to survive. Unicellular organisms from the genus *Acanthamoeba* have evolved strategies to extend the range of the suitable growth conditions and to survive unfavourable conditions. These strategies include process of encystation, which produces resistant cysts that remain viable for months or years, and formation of pseudocysts, a rapid synthesis of a thin protective coat that protect the cell for several days, observed experimentally after exposure of acanthamoebae to organic solvents.

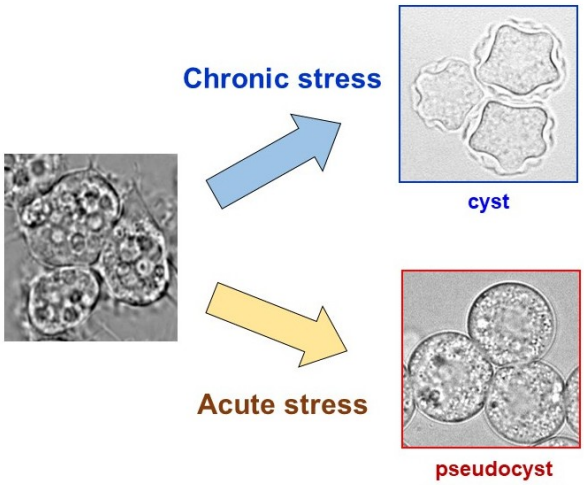

Fig. 4 The cyst and the pseudocyst.

1.4.1. Conditions leading to encystation vs. pseudocyst formation

1.4.1.1. Encystation.

Encystation, i.e., differentiation of trophozoites into the dormant cyst stage covered with a double-layered cyst wall, occurs under long term stress caused by fundamental changes in some of standard living/favourable conditions, e.g., a lack of nutrients, pH and temperature extremes, long term hypoxia, as well as some disinfectants or drugs (Neff and Neff, 1969; Band and Mohrlok, 1973; Cordingly et al., 1996; Bergmanson et al., 2011). To induce encystation in laboratory setting, several methods were described (Neff et al., 1964; Bowers and Korn, 1969; Weisman, 1976; Schuster, 2002; Lloyd, 2014), of which (Lloyd, 2014) addition of 50mM $MgCl_2$ to PYG culture medium or nutrient starvation (Neff et al., 1964) are used most frequently (Khan 2006). Spontaneously, without external manipulation with culture

conditions, acanthamoebae encyst when cultivating on the non-nutrient agar covered with heat-inactivated bacteria as a source of food. Cysts occur after feeding the bacteria (Bowers and Korn, 1969; Weisman, 1976; Schuster, 2002) as well as in stationary cultures under axenic conditions (Neff et al., 1964; Lloyd, 2014). Interestingly, definitive explanation of what constitutes the actual trigger of encystation is so far lacking. Proposed factors include high osmolarity (Cordingley et al., 1996; Dudley et al., 2005), Ca^{2+} or Mg^{2+} ions and decreased oxygen levels (Weisman, 1976; Turner et al., 1997).

1.4.1.2. Pseudocyst formation

Formation of *Acanthamoeba* pseudocysts, i.e., differentiation of trophozoites into a round dormant stage covered with filamentous surface coat occurs under acute stress originally simulated by exposure of trophozoites to organic solvents – methanol, acetone and DMSO in vitro (Kliescikova et al. 2011a). The pseudocysts formation was also described in the *Acanthamoeba* trophozoites exposed to propylene glycol (Kliescikova et al. 2011b), polyhexamethylene biguanide (Huang et al. 2017) and chlorine disinfection solution (Sarink et al. 2020). Moreover, similar stages were also found in a marine amphizoic amoeba, *Neoparamoeba perurans* (Lima et al. 2017; Botwright et al. 2020). In vitro, the *Acanthamoeba* trophozoites exposed to organic solvents in concentrations ranging from 0.1% to 10% at 37^0C differentiate into mature pseudocyst within 2 hours. In lower temperature, higher solvent concentration was needed and the response time extended (Kliescikova et al. 2011a).

1.4.2. Viability

In contrast to long-term living cysts (Mazur et al., 1995; Campbell et al., 2008; Axelsson-Osson et al., 2009; Aksozek et al. 2002; Sriram et al., 2008), the pseudocysts remain viable for one week (Klieščikova et al., 2011a). After transfer into a solvent-free growth medium, the pseudocysts promptly (within 2 h) transform into amoeboid trophozoites leaving their coat behind. Similarly, in the presence of encystation medium lacking the solvent cells transform into trophozoites and only then begin the encystation (Kliescikova et al. 2011). The process of encystation is, compared to pseudocyst formation, quite slow: it takes approximately 16 - 24 hours to produce mature cysts with fully developed double-layered wall (Neff et al. 1964; Lloyd et al., 2001; Hughes et al. 2003; Köhsler et al., 2008; Klieščikova et al., 2011ab). The process of excystment is initiated within minutes after transfer of cysts to favourable conditions. The full emergence of amoebae requires about 24 hours from initiation. Only

about 3 minutes are needed for the passage of the organism through the cyst ostiole (Mattar and Byers 1971).

Based on the above-described differences it is assumed that encystation helps *Acanthamoeba* to survive relatively slowly coming, long term and unfavourable conditions. On the other hand, pseudocyts are formed upon acute stressful incidents to protect the cell for a shorter time but immediately. However, in regard to this it should be noted that it is not known if pseudocysts are formed also in the environment, what is the full spectrum of compounds able to trigger this reaction and how long pseudocysts can stay alive and what affects their survival. On the other hand, a definitely established fact is that the pseudocyst formation is not fixed to the T4 genotype but was documented also in T1, T5, T6 and T11 genotypes (Klieščikova et al., 2011b).

1.4.3. Ultrastructural and molecular differences

At the ultrastructural level, the most pronounced difference between the cyst and the pseudocyst is found in the surface envelopes of both stages. On a surface of the mature pseudocyst there is a single-layered finely fibrillar coat resembling glycocalyx. The coat with a thickness of approximately 80-100 nm externally covers the plasma membrane. The coat is evident on the surface of rounded cells already after 30 minutes after initiation of pseudocyst formation. Coat material is deposited on the surface through fusion of cytoplasmic tubules with the plasma membrane. There are two types of the mature pseudocysts identified by transmission electron microscopy TEM, pseudocysts with smooth plasma membranes and those with wrinkled plasma membranes (Klieščikova et al., 2011a), although the functional consequence of the morphological differences is as yet not understood.

The surface of a mature cyst is covered by a double-layered wall, 5–20 μm thick. The outer cyst wall, the ectocyst is wrinkled and the inner cyst wall, the endocyst is polygonal with a tendency to spherical shape. There are diference in thickness where endocyst has been showed to be thicker (Garajova et al. 2019).

The first to form is ectocyst layer. It is wrinkled with folds and ripples and contains proteins and lipids (Bowers and Korn, 1969; Weisman, 1976). In contrast to the texture of the pseudocyst coat, the amorphous ultrastructure of the early ectocyst exhibits a markedly distinct pattern. It is composed of irregularly dense layers of amorphous material deposited externally on the plasma membrane (Klieščikova et al., 2011a). The endocyst consists of at least three distinguishable layers where the most external one is composed of cellulose microfibrils. Both walls are separated, except at pores and ostioles that are the points of

communication of the acanthamoba cyst with the external environment. (Bowers and Korn, 1969; Weisman, 1976; Garajova et al. 2019).

Regarding the organelles, pseudocyst seems to maintain all of the organels in more or less unchanged shape and number. The cytoplasm of immature pseudocysts (at 1 hour after methanol exposure) contains many mitochondria and lysosomes as well as prominent cisternae of rough endoplasmatic reticulum and a pulsating vacuole with a vesicular system. In mature pseudocyst there are still clearly evident many mitochondria and the nucleus with a large nucleolus (Klieščikova et al., 2011a).

In contrast, encysting *Acanthamoeba* undergoes a large change in number and composition of organelles. There is more than a 50% reduction of the cell volume which means that many of the organelles are reduced by autolysosomes which are present in a large number during the whole process of encystation (Bowers and Korn, 1969). The digestive vacuoles disappear during later stages of encystation and their content is discharged. In the mature cysts many lysosomes and peroxisomes are present (Muller, 1969; Muller and Moller, 1969), whereas mitochondria are condensed and incorporated into autolysosomes (Bowers and Korn, 1969).

So far, limited information is available about both encystation and the pseudocyst formation at the molecular level. Nevertheless, several genes for synthetic pathways of proteins involved in the encystation process have been proposed. These include cellulose synthase (Potter and Weisman 1971), adenylate cyclase (Achar and Weismann 1980), polyphenol oxidase (Sykes and Band 1985), zinc finger protein, transcription factor IIIA (Matthews et al., 1995), cyst specific protein (Hirukawa et al. 1998), protein kinase C, Na P-type ATPase (Moon et al. 2007), heat shock protein, cullin 4 and encystation-mediating serine protease (Moon et al., 2008), autophagy protein 8 (Moon et al. 2009), serine protease (Dudley et al. 2008), cysteine protease (Leitsch et al., 2010; Moon et al. 2012), xylose isomerase (Aqeel et al. 2013) and cyst wall proteins (CWPs) by three families of lectins (Magistrado-Coxen et al., 2019) and others (see Tab 1).

Several of the encystations-associated protein were analyzed also in relation to pseudocysts formation by Klieščikova et al., (2011a) and this work represents the only relevant refence to this topic so far. In this publication, glycogen phosphorylase (GP), CSI (cellulose synthase I), CSII (cellulose synthase II) were analyzed. It was found that all three genes were expressed during the process but at different patterns. The expression was highest at 30 resp. 60 minutes after induction of pseudocyst formation compared to encystation, where the expression seems to be relatively constant during the whole process. Interestingly,

no signal was obtained for cyst-specific protein 21 (CSP21), recognised as a typical protein for encysting cell populations.

1.4.4. Differences in resistance to physical and chemical stimuli

As can be expected, regarding cyst vs. pseudocyst surface coat thickness, pseudocysts are less resistant than cysts. In the presence of heat viability of the mature cysts diminish after 10 min at 65 °C. Pseudocysts are resistant to a 10-min exposure to 55 °C, whereas higher temperatures are lethal and caused granulation of their cytoplasm. Pseudocysts are resistant to alkaline pH (8-11), but not to acidic pH values (3-5), compared to the cysts which are resistant to the entire range of pH values. Although mature cysts are able to survive for years (Aksozek et al. 2002; Mazur et al. 1995; Sriram et al. 2008) time of pseudocyst survival in the external environment is not known. It was proven by Klieščikova et al., (2011a) that pseudocysts are able to survive 7 days without water but data with longer period of time are lacking.

In summary, it is obvious that the cyst and the pseudocyst represent distinct and well-defined stages of the *Acanthamoeba* life cycle while many questions remain as to the molecular mechanisms of the development of the resistant stages, their relation to the cell cycle and mechanistic explanation of the triggers of these defence reactions. Apparently, this also includes the issue of existence of molecular mechanisms shared between the two processes.

1.4.5. Effort to inhibit *Acanthamoeba* encystation

With the access to new molecular biological methods together with whole *Acanthamoeba* genome(s) publication, new possibilities how to explore the phenomenon of encystation have arisen. There are publications addressing *Acanthamoeba* encystation resp. inhibition of the progress of encystation by targeting various proteins which are believed to participate on the differentiation process (Tab. 1). The most commonly used method is the RNA interference by using small interfering RNA (siRNA) which can by enzyme complexes degrade messenger RNA (mRNA) and thus decrease the production of target gene product (Dana et al., 2017). The reported efficiency of such a treatment on the encysting *Acanthamoeba* cell population vary, according to various authors, between 10% -80%. The problem which none of the works have discussed, although it potentially represents a significant liability, is that, at least some of them, determined the pseudocysts as an incomplete encystation or failure of the encystation process. Support for this assertion can indeed be found in the photographic documentation

given in the respective publications (Moon et al., 2009, 2012, 2013, 2014, 2016; Song et al., 2012; Kim et al., 2015; Lee et al., 2015). Consequently, the outstanding questions to be considered are: is the process of gene silencing by siRNA interference indeed effective or can the application of various transfection protocols, including using various siRNA transfection reagents, lead to pseudocyst formation instead? Did the strains of *Acanthamoeba* used in the studies maintain the ability to encyst (many of authors demonstrated that after prolonged

Tab 1: Publications from last two decades addressing *Acanthamoeba* encystation by targeting various methabolic pathways and proteins by using mainly molecular biology techniques.

Author	**Year of publication**	**Target protein or biochemical process**	**Method**
Morales et al.	2008	Glycogen Phosphorylase	siRNA silencing
Moon et al.	2009	Autophagy protein 8	siRNA silencing
Aqeel et al.	2012	Xylose isomerase, cellulose synthase	siRNA silencing
Moon et al.	2012	Cysteine protease	siRNA silencing
Moon et al.	2012	Protein kinase C	siRNA silencing
Song et al.	2012	Autophagy protein 16	siRNA silencing
Lee et al.	2013	Cysteine protease inhibitor AcStefin	siRNA silencing
Moon et al.	2013	Autophagy-related protein 8 (Atg8)	siRNA silencing
Du et al.	2014	cAMP phosphodiesterase RegA	treatment by inhibitors
Jha et al.	2014	Chlorochine modulate autophagy	treatment by inhibitor
Moon et al.	2014	Cellulose synthase	siRNA silencing
Kim et al.	2015	Autophagy protein 12	siRNA silencing
Lee et al.	2015	M17 Leucine Aminopeptidase	siRNA silencing
Moon et al.	2015	Cellulose synthesis inhybitors	treatment by inhibitors
Moon et al.	2015	Autophagy inhibitors	treatment by inhibitors
Moon et al.	2016	Methyltransferase 5	siRNA silencing
Moon et al.	2017	DNA methyltransferase	treatment by inhibitor
Rolland et al.	2020	putative N-acetyltransferase	transfection by plasmid

cultivation the strains lose their encystment potential)? Moreover, only several authors paid attention to control the efficiency of the siRNA silencing method by using RT-qPCR with the appropriate reference genes as mentioned by Köhsler et al. (2020).

2. Aims of thesis

This PhD thesis aims at highlighting the awareness of the pseudocyst existence and deepen the understating of differences between encystation and pseudocyst formation on molecular level and together with better characterization both stress defence reactions. The specific issues addressed are:

I. To identify genes of metabolic pathways involved in synthesis of protective sugars, trehalose and mannitol in *Acanthamoeba* genome and to perform the phylogenetic and functional analysis of the protein sequences.

II. To determine the role of trehalose and mannitol in *Acanthamoeba* protection, especially during encystation and pseudocyst formation and in the dormant stages cyst and pseudocyst.

III. To identify the point within the cell cycle from which *Acanthamoeba* enters encystation and pseudocyst formation and what is the DNA content of the mature cysts and the mature pseudocysts.

3. Results, discussion and perspectives

The present thesis is based on following publications:

I. Bínová E, Klieščiková J, Ashford D, Thomas-Oates J, Nohýnková E (2012). Mannitol is not involved in protective reactions of *Acanthamoeba*. Molecular and Biochemical Parasitology 184:118-121

II. Bínová E, Bína D, Ashford DA, Thomas-Oates J, Nohýnková E (2017). Trehalose during two stress responses in *Acanthamoeba*: Differentiation between encystation and pseudocyst formation. Protist 168:649-662

III. Bínová E, Bína D, Nohýnková E (2020). DNA content in *Acanthamoeba* during two stress defense reactions: Encystation, pseudocyst formation and cell cycle. European Journal of Protistology 77:125745 doi: 10.1016/j.ejop.2020.125745.

3.1. Publication I

Mannitol is the most abundant six-carbon acyclic sugar alcohol/polyol occurring in nature. It is known to be synthesized and used as a carbohydrate storage, translocatable assimilate, a source of reducing power, an osmoregulator and/or antioxidant by various organisms from bacteria to higher plants (Stoop at al., 1996; Wisselink et al., 2002). Various organisms accumulate this polyol in connection to transformation into resistant and/or reproductive stages, spores and oocysts (Schmatz, 1997; Ruijter et al., 2003). In our study we focused on mannitol as a possible protective agent helping *Acanthamoeba* to protect the inner space of the dormant stages, the cyst and pseudocyst.

To find out the amounts of mannitol in *Acanthameoba* trophozoites, cysts and pseudocysts we performed the hydrophilic interaction liquid chromatography (HILIC) coupled to negative ion mode electrospray ionisation mass spectrometry (ESI-MS). The results were rather surprising because no mannitol was revealed in any *Acanthamoeba* stage, although the genome studies by Watkins and Gray (2008) published sequences for both genes of mannitol synthetic pathway, mannitol phosphate dehydrogenase (MPDH) and mannitol dehydrogenase (MDH). We did not find mannitol but our search revealed other less usual sugars in *Acanthamoeba* cysts, pseudocysts and trophozoites. Cysts and pseudocysts contained a disaccharide trehalose. In pseudocysts there was also maltose (the role of trehalose, and also maltose, in *Acanthamoeba* focused our next publication), whereas in cysts we detected trisaccharide raffinose. Raffinose is an interesting sugar in that it is found mainly in plants, where it plays a role in the desiccation tolerance of seeds (Saravitz et al., 1987; Castillo et al., 1990). It also protects liposomes from dehydration (Hincha et al., 2003). It was

reported that the expression of enzymes related to the biosynthesis of raffinose and their intracellular accumulation in plant cells are closely associated with the responses to environmental stresses (Taji et al., 2002; Kaplan et al., 2004, 2007; Panikulangara et al., 2004; Peters et al., 2007, Nishizawa et al., 2008). Its presence in the *Acanthamoeba* cysts can be also connected with the cell protection during unfavourable environmental conditions.

Because our ESI-MS data did not reveal the presence of mannitol in any stage of *Acanthamoeba*, we analysed in detail the two sequences of mannitol synthetic pathways annotated by Whatkins and Gray (2008) as MPDH and MDH. Our analysis revealed the possible misinterpretation of previously published sequences as alcohol dehydrogenase sequences different from mannitol dehydrogenases.

By using qRT-PCR, we have compared mRNA levels of these enzymes at various time intervals during the stress defense reactions of encystation and pseudocyst formation and we found out that whereas during encystation the levels of mRNA for the enzymes decrease, during formation of the pseudocyst, especially at the beginning of the process, mRNA levels increase in both enzymes. This demonstrates that the pseudocyst is at the beginning very metabolically active, not only in the cellulose synthase and glycogen phosphorylase levels (Klieščiková et al., 2011) but also in metabolism of sugar alcohols.

3.2. Publication II

In many eukaryotes, stress resistance is supported by the synthesis of protective compounds to alleviate the effects of anhydrobiosis, freezing, and osmotic pressure on macromolecular assemblies such as membranes. This role is often played by carbohydrates such as trehalose or mannitol (Lourenço et al. 2016). In this study, we searched the *Acanthamoeba* genome for putative enzymes involved in trehalose synthesis pathways and determined correlations between the mRNA expression patterns of the enzymes and concentrations of cellular carbohydrate pools during encystation and pseudocyst formation.

There are at least five different pathways for trehalose synthesis described in bacteria and archea (Avonce et al. 2006, Paul et al. 2008). Only one of them is known from eukaryotes (Roth and Sussman 1966). Our analysis of the *Acanthamoeba* genome revealed sequences for enzymes from two different pathways of trehalose synthesis. The eukaryotic TPP/TPS pathway and the bacterial TreS pathway. While the presence of the TPS-TPP enzymes could be expected, the presence of the prokaryotic TreS pathway was somewhat unique and surprising. Our BLAST search based on the *A. castellanii* sequence showed the presence of putative TreS sequences also among other species of free-living Amoebozoa. The monophyly

in the basal position, with respect to the bulk of prokaryotic proteins, suggests a single gene transfer event preceding the diversification of the extant Amoebozoa members. Such placement of the eukaryotic TreS might be also a result of faster molecular evolution of bacteria than eukaryotes, leading to faster divergence of the bacterial sequences. This possibility implies that the eukaryotic TreS sequence might in fact represent a more primitive form of the protein. Comparative analysis of the Amoebozoa TreS with the mycobacterial enzymes together with the conservation of the catalytic residues and the domain organization of the enzyme indicate that the TreS sequence recovered from the *Acanthamoeba* genome represents an active enzyme. The phylogenetic analysis of the three fused TPS/TPP enzyme sequences found in *Acanthamoeba* genome was clearly consistent with the findings of Avonce et al. (2010) who did the analysis for the TPS/TPP protein sequences of the amoebozoan slime mold *Dictyostelium discoideum*. They divided the protein sequences into two separate groups of enzymes, one associated closely with prokaryotic fused (TPS-TPP) enzymes and the other on the basis of the fungal sequences.

The genomes of many organisms contain multiple copies of the enzymes of the TPS-TPP pathway, with many of these copies likely coding for enzymatically inactive proteins serving regulatory or structural functions (Avonce et al., 2006). Thus, we analyzed the sequences of the TPS-TPP from *A. castellanii* in more detail to assess their functionality. We found out that there is no fully active TPS/TPP fusion enzyme. There is only one clearly active TPS and two TPP enzymes from distinct groups. Nevertheless, the two phylogenetically distinct types of TPS-TPP appear to be able to complement each other in providing the complete pathway of trehalose synthesis in *Acanthamoeba*.

We showed that genes for all four trehalose biosynthetic enzymes found in the *Acanthamoeba* genome are expressed during both types of stress reactions but with some differences. During formation of the pseudocysts, we observed the most pronounced increase in mRNA expression for all four genes within the first 30 minutes whereas we detected upregulation in only two of the four genes of the enzymes for trehalose synthesis, TreS and one TPS-TPP, during *Acanthamoeba* encystation. Also, the amounts of mRNA levels differ very rapidly and considerably. These different expression patterns of the enzymes for trehalose synthesis pathways during encystation and pseudocyst formation highlight the dissimilarities of both processes as mentioned already by Klieščiková et al., (2011).

It seemed that in the early/immature pseudocysts (first 30 minutes of the pseudocyst formation process), TreS predominates in the synthesis of trehalose since the increase in the amount of trehalose apparently correlates with a decrease in maltose, a substrate for trehalose

synthesis in the TreS pathway (Nishimoto et al. 1995). The function of trehalose in this phase of *Acanthamoeba* differentiation is unknown. In general, trehalose may serve as an energy and carbon source, a signaling or regulation molecule, a cell wall component, or a membrane and protein protectant (Elbein et al. 2003). A significant decrease in trehalose in the maturing pseudocysts indicates the former possibility. At the same time, a role for the enzymes of the TPS-TPP pathway remains completely unclear (provided that all the trehalose detected at the 30-minute time point was formed by converting maltose via TreS, the TPS-TPP pathways could not be involved in trehalose synthesis). On the other hand, the activities of these enzymes may yield other important molecules. For instance in plants, trehalose-6-phosphate (T6P), the metabolic precursor of trehalose in the TPS-TPP biosynthetic pathway, is an important signalling metabolite (O´Hara et al. 2013). It seems that in the mature pseudocysts, trehalose is formed particularly via the TPS-TPP pathways, because the concentration of maltose is close to zero at this time. The function of trehalose in mature pseudocysts could be connected with the need for compounds that protect the inner environment from the deleterious effect of thermal and drought stress.

During encystation, the TreS pathway seemed to play a major role in trehalose synthesis since TreS was found to have the highest expression level of mRNA and since an increase in trehalose was mirrored by a decrease in maltose. However, a comparison of the levels of the two disaccharides clearly shows that maltose could not be the only substrate for trehalose synthesis. This observation suggests that either one of the TPS-TPP represents a fully active enzyme capable of catalyzing both steps of the trehalose synthesis, despite the amino acid substitutions, or that the TPS-TPP acts as a part of a multi-component complex, either by providing the TPP activity or playing a regulatory role, similar to Tsl1 and Tps3 in *Saccharomyces* (Gancedo and Flores 2004).

Gradual decrease in the amount of trehalose after 24 hours of encystation indicating a partial breakdown of the disaccharide within the mature cysts. As mature *Acanthamoeba* cysts represent highly resistant non-motile cells (thanks to a fully developed double-layered cyst wall) with a reduced total volume, dense cytoplasm due to dehydration by water expulsion, fewer organelles in comparison with trophozoites and minimal metabolic activity (Bowers and Korn 1969) they are very limited in energy resources; they cannot use exogenous nutrients, and endogenous energy supplies, especially glycogen, are mostly depleted for cyst-wall synthesis or are excreted into the outer space of the encysting cell during autophagy (Khan 2009). Thus, one possibility is that trehalose hydrolysis could provide an energy or

carbon source in the mature cysts. Trehalose degradation may also be necessary to stabilize the internal osmolarity of long-living *Acanthamoeba* cysts.

To summarize, we have detected the presence of the prokaryotic TreS pathway in a eukaryotic *A. castellanii* during both encystation and pseudocyst formation. The active apparatus for trehalose synthesis and the presence of a large amount of trehalose during both reactions point to an important role for trehalose in *Acanthamoeba* stress responses. A precise determination of the function of this disaccharide however remains to be done in the future. Application of methods of genetic transformation together with modern biochemical methods, which are now in *Acanthamoeba* research very rare and difficult to perform, would be necessary.

3.3. Publication III

In general, cell differentiation is believed to be closely associated with the cell cycle regulation. Before the onset of differentiation, cells usually exit the cell cycle from a specific point during the gap (G) phase. In our work we determined the cell cycle position from which the trophozoites commence encystation and pseudocyst formation.

To explore processes related to the cell cycle it is usual to work with cell population synchronized and stopped at a specific phase of the cycle. To this end we chose two commonly used cell cycle inhibitors, aphidicolin (APH) and hydroxyurea (HU) that halt eukaryotic cells at the G1/S boundary through the inhibition of DNA replication (Bucknall et al., 1973; Yarbro, 1992). Surprisingly, neither APH nor HU stopped the *Acanthamoeba* trophozoite populations in a defined phase of the cell cycle.

In case of APH, which blocks DNA replication by competing with deoxynucleotides (dNTPs) to bind to eukaryotic DNA polymerase alpha (Huberman, 1981; Spadari et al., 1982), we also observed nearly no effect on the growth of the trophozoites. Hence, we examined whether the target of the inhibitor, DNA polymerase alpha is present in the *Acanthamoeba* genome and upon confirming this we carried out closer investigation of the protein sequence of the enzyme for possible hints of its resistance to APH. However, no apparent difference of the *Acanthamoeba* enzyme compared to sequence of other organisms in which the use of APH for cell synchronization is well established (Baranovskiy et al., 2014) was found. Thus, other reasons for the failure of APH on *Acanthamoeba* have to be considered. One of potential candidates is the oxidative metabolism, by the action of cytochrome P450, which is known to efficiently inactivate APH, as it is known in animal and

human systems (Edelson et al., 1990). Worthy of consideration is the fact that APH is a compound produced by a number of soil fungi (Bucknall et al., 1973), thus the ability to metabolize it can be considered advantageous for a free-living soil inhabiting protist such as *Acanthamoeba*.

The issue of the failure of synchronization of the cells on the G1/S boundary is more comlex in the casae of HU, because we obtained results showed a dose-dependent influence of HU on *Acanthamoeba* growth, which indicate a presence of some effect on *Acanthamoeba* cell population. Our investigation of *Acanthamoeba* genome confirmed the presence of the protein sequences of both subunits of ribonucleotide reductase (RNR), Class I, a HU target, as it has been found by Clarke et al. (2013). However, a more extensive search revealed the presence of another RNR, namely, RNR from Class II. This type of RNR, present mainly in Archaea and Eubacteria, is completely oxygen-independent and uses coenzyme B12 (adenosylcobalamin) as a metal cofactor (Torrents, 2014) and is not affected by HU. Hence, the presence of the Class II RNR in the *Acanthamoeba* genome, could be the reason for the failure of HU to synchronize the *Acanthamoeba* trophozoites but less so for the effect on the *Acanthamoeba* growth. Since it is known that HU can cause DNA damage and so activate the DNA damage checkpoints which slow down the cell population growth (Weinberger et al., 1999), we performed experiment in which we used antibody against phosphorylated histone H2A, a phosphorylated variant of H2AX histone that plays central role in DNA damage signalling in eukaryotic cells sharing the SQ motif on C-tail (Georgoulis et al. 2017; Zhou and Elledge 2000), to investigate the rate of DNA damaged cells. We observed dose and time-dependent effect on *Acanthamoeba* trophozoites after HU treatment (data not shown). These results lead us to propose that the effect of HU on the *Acanthamoeba* growth is explained by a slowdown of their multiplication as a consequence of DNA damage and the urgency of DNA repairs, rather than cell synchronization.

As we were not able to achieve synchrony in *Acanthamoeba* cell population, we followed our investigations by analysis of the non-synchonized *Acanthamoeba* populations. By using the method of flow cytometry to estimate DNA content we observed in the population of exponentially growing *Acanthamoeba* trophozoites two characteristic peaks, one corresponded to cells in the G1 cell cycle phase, and the second peak to G2 phase. Because our observation was in contrast to works of other authors (Band and Mohrlok 1973; Edwards and Lloyd, 1978, 1980; Stöhr et al., 1987; Jantzen et al., 1990; Byers et al., 1991) it led us to suggestion that the discrepancies might result from dissimilarities in the assays used for the DNA content studies. This methodological problem was mentioned also by Byers et al.

(1991) in the most recent study on this topic. Regarding this, our data can be considered very robust due to application of modern method for measuring DNA content (Darzynkiewicz et al., 2010).

Our flow cytometry analysis focused on processes of *Acanthamoeba* encystation and pseudocyst formation revealed that *Acanthamoeba* enters encystation from the G2 phase of the cell cycle but differentiation into pseudocysts can begin in the G1 and also in G2 phases. However, the DNA content of the mature cysts and pseudocysts is the same, with doubled DNA. In contrast to encystation, pseudocyst formation is a fast process finished within 2 hours. It responds to acute environmental stress factors when it is of utmost importance to enable the cell to escape the acute life-threatening conditions. The cell at the beginning of pseudocyst formation are much more metabolically active that the encysting cells (Bínová et al., 2017, Section 3.2.). The short timescale on which this differentiation process occurs is consistent with the previous suggestion of Kliescikova et al. (2011a) that *Acanthamoeba* cells can begin pseudocyst formation regardless of their presence in the G1 or G2 phases of the cell cycle. Our finding that *Acanthamoeba* differentiates into cysts only from the G2 phase of the cell cycle is in agreement with results of authors studying the DNA content in dormant stages of other protists e.g., the parasitic amoeba *Entamoeba invadens* (Ganguly and Lohia, 2001) or the parasitic flagellate *G. intestinalis* (Bernander, 2001; Reiner et al., 2008). Our results also suggest the importance of the preservation of genome integrity during the formation of resistant resting stages in *Acanthamoeba*e. Apparently, the risk of losing important cellular function due to random mutation is reduced in this case (Kermi et al., 2019). Moreover, the presence of more copies of genes encoding components of metabolic machinery may allow for the faster initiation of cellular processes upon conversion of the resting stages back to the proliferating, vegetative amoebae and consequently the rapid colonization of the environment, as suggested for other Amoebozoan protist, namely the soil-dwelling social amoeba *Dictiostelium discoideum* (Muramoto and Chubb, 2008).

Hence, to obtain further insight into *Acanthamoeba* cell cycle regulation we performed an analysis of the *Acanthamoeba* genome with respect to the pool of cyclin-dependent kinases (CDKs) and cyclins, which are the main regulators of eukaryotic cell cycle progression. Results of our findings suggests a remarkable simplicity of the cell cycle regulatory machinery in *Acanthamoeba*. Our analysis suggested presence of only one CDK related to human CDK1 and yeast Cdc28, in agreement with previous work of Mengue et al. (2016), also compared to this publication, our analysis was based on a broader phylogenetic sample of CDK sequences. Further, we inferred the absence of cyclins of the D/E type which activate

Cdk4/6 and Cdk2, respectively, and play a role in the G1-S transition. Given the absence of both bona fide Cdk4/6 and Cdk2 in Amoebozoa, the lack of their respective cyclin partners is not surprising. Moreover, it should be noted that Cdk4/6, Cdk2 and their associated D/E cyclins appear to be nonessential for entry into the cell cycle, even in mammalian cells (Malumbres and Barbacid, 2009). On the other hand, *A. castellanii* contains three Cdk9-like sequences. Cdk9 was reported to play a role in the replication stress response in human cells (Yu et al., 2010), but whether the Cdk9-like sequences performs the same role in *A. castellanii* and thus whether the increase in the number of Cdk 9 homologues is related to this function has yet to be investigated.

The present study answered several questions mentioned the *Acanthamoeba* cell cycle, especially the position from which enters encystation and pseudocyst formation as the two fundamental processes its life cycle. We also came across some interesting questions which remain to be addressed in the future, for example the functions of the two RNRs and the issue of *Acanthamoeba* response to DNA damage.

4. Conclusions

I. Genes coding for enzymes of trehalose synthesis are present in the genome of *A. castellanii*. These represent the TPS-TPP-based pathway widespread among eukaryots but also the prokaryotic TreS pathway. Detailed analysis of the gene sequences indicate that both trehalose synthetic pathways are functional. On the other hand, present analysis suggests that contrary to previously published data, enzymatic machinery for production of the protective polyol mannitol is not present.

II. In agreement with the analysis of protein sequence data, trehalose is present in all known forms of *Acanthamoeba,* i.e., the vegetative trophozoites, pseudocysts and cysts, whereas mannitol was not identified in any of the *Acanthameoba* life forms. There are quantitative changes in the amount of trehalose during encystation as well as pseudocyst formation with higher concentrations of the sugar at the beginning of the processes. This suggests a protective role of trehalose in *Acanthamoeba*, especially under stress conditions.

III. Asynchronous population of *Acanthamoeba* trophozoites in logarithmic growth phase comprises cells residing both in G1 and G2 phase of the cell cycle, as evidenced a bimodal distribution of DNA content as observed by flow cytometry. *Acanthamoeba* enters encystation from the G2 phase of its cell cycle, whereas it differentiates into pseudocysts from both the G1 and G2 phases of the cell cycle. Nevertheless, the DNA content of both mature cysts and pseudocysts is the same, indicating that the cells survive unfavourable environmental conditions with replicated genomes.

Declaration of the student participation on publications

I declare that Eva Bínová participated on preparation of all papers representing a basis of her PhD Thesis as follows: paper I. - 65% contribution (co-designed the experiments, performed qRT PCR, bioinformatic analysis, wrote the manuscript); paper II. – 65% contribution (designed experiments, performed qRT PCR, mass spectrometry data analysis, participated in phylogenetic analysis, wrote the manuscript); paper III. – 75% contribution (conceptualization, performed FACS, inhibition tests, formal analysis, wrote the original draft).

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6. Appendix

6.1. **Bínová E, Klieščiková J, Ashford D, Thomas-Oates J, Nohýnková E (2012)**. Mannitol is not involved in protective reactions of *Acanthamoeba*. Molecular and Biochemical Parasitology 184:118-121

6.2. **Bínová E, Bína D, Ashford DA, Thomas-Oates J, Nohýnková E (2017).** Trehalose during two stress responses in *Acanthamoeba*: Differentiation between encystation and pseudocyst formation. Protist 168:649-662

6.3. **Bínová E, Bína D, Nohýnková E (2020).** DNA content in *Acanthamoeba* during two stress defense reactions: Encystation, pseudocyst formation and cell cycle. European Journal of Protistology 77:125745 doi: 10.1016/j.ejop.2020.125745.