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Role of ABC transporter in drug resistance of *Acanthamoeba castellanii*.

Úloha ABC transportéru v lékové rezistenci *Acanthamoeba castellanii*.

Diploma thesis

Supervisor: RNDr. Jan Mach, Ph.D.

Prague, 2025

Prohlášení:

Prohlašuji, že jsem závěrečnou práci zpracovala samostatně a že jsem uvedla všechny použité informační zdroje a literaturu. Tato práce ani její podstatná část nebyla předložena k získání jiného nebo stejného akademického titulu. Při sepisování práce jsem použila umělou inteligenci či nástroje jí podporované, a to následovně: ChatGPT pro jazykovou korekci, DeepL pro jazykovou korekci.

V Praze dne 6.8. 2025 .....…….

Eliška Drncová

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Abstract

The ABC transporter protein family holds one of the key roles in drug resistance of a plethora of human pathogens. The ABC transporters partake in cellular efflux of various substrates, like drugs. Their function, also known as multi drug resistance (MDR), complicates the treatment of many diseases including pathogens from every domain of life. A unicellular human parasite *Acanthamoeba castellanii*, the cause of acanthamoeba keratitis and granulomatous acanthamoeba encephalitis, eludes most treatments, which results in a poor prognosis. A recently identified ABC transporter was found to partake in the response to oxidative stress, induced by PEITC and rotenone in *A. castellanii*. Herein, its presumed role in drug resistance was investigated utilizing molecular techniques to characterise the functional properties of this ABC transporter. The response of the ABC transporter to PEITC, rotenone and amphotericin B was observed in a transcription analysis. Subsequently. a partial knockout strain was produced, and showed a promising phenotype in PEITC, suggesting the involvement of the ABC transporter in PEITC detoxification. Furthermore, based on the acquired data we propose, that more substrate specific ABC transporters are involved in the drug resistance of *A. castellanii.* However, the knockout method was proven to be insufficient, thus a new CRISPR Cas9 method was conducted, one that could facilitate the production of a knockout strain of multiple ABC transporter paralogs. The knowledge of *A. castellanii* MDR is limited, therefore, this research could shed light on the emergence of resistance in *A. castellanii* and improve the efficiency of treatment.

Key words: *Acanthamoeba castellanii*, ABC transporter, drug resistance, knockout, cellular efflux, PEITC, amphotericin B

Abstrakt

Jednu z klíčových rolí v lékové rezistenci mnoha lidských patogenů zastávají proteiny z rodiny ABC transportérů. ABC transportéry se podílejí na exportu látek, mimo jiné i léčiv, z buňky. Jejich působení tak komplikuje léčbu některých infekčních onemocnění. Tento jev, také známý jako mnohočetná léková rezistence neboli multi drug resistance (MDR), se vyskytuje u organismů ze všech domén života. Jednobuněčný lidský parazit *Acanthamoeba castellanii*, který způsobuje akantamébovou keratitidu nebo granulomatózní akantamébovou encefalitidu, je odolný vůči většině druhů léčby a může za nepříznivou prognózou u pacientů. Nově objevený ABC transportér u *A. castellanii* působí během odpovědi na oxidativní stres, který byl vyvolán látkami PEITC a rotenone. Pomocí molekulárních metod byla v této práci zkoumána jeho funkce a role v lékové rezistenci. Použitím transkripční analýzy byla sledována odpověď ABC transportéru na látky PEITC, rotenone a amphotericin B a následně byl u vytvořené částečné knockout linie sledován fenotyp v přítomnosti léčiv. Vystavením látce PEITC se projevil fenotyp naznačující účast ABC transportéru na její detoxifikaci. Získaná data vedou k hypotéze o zapojení více ABC transportérů, lišících se specifitou pro substrát, do lékové rezistence *A. castellanii*. Metoda použitá pro zhotovení knockout linie se ukázala jako nedostačující, proto byla zavedena metoda CRISPR Cas9, která byla nedávno poprvé použita u *A. castellanii* a mohla by vést k vytvoření linie s odstraněnými geny pro několik paralogů ABC transportérů. Povědomí o MDR u *A. castellanii* je stále omezené, pokračování tohoto výzkumu tak má potenciál rozluštit otázku vzniku rezistence u *A. castellanii* a zvýšit účinnost léčby.

Klíčová slova: *Acanthamoeba castellanii*, ABC transportér, léková resistence, knockout, buněčný eflux, PEITC, amphotericin B

Abbreviations

Ab	antibody
ABC	ATP binging cassette
ADP	adenosine diphosphate
AK	acanthamoeba keratitis
ATP	adenosine triphosphate
CFTR	cystic fibrosis transmembrane conductor
DNA	deoxyribonuclease acid
GAE	granulomatous acanthamoeba encephalitis
GAPDH	glyceraldehydephosphate dehydrogenase
GFP	green fluorescent protein
IPTG	isopropyl β-d-1-thiogalactopyranoside
KO	knockout
MDR	multi drug resistance
NBD	nucleotide binding domain
OE	overexpression
PCR	polymerase chain reaction
PEITC	phenethyl isothiocyanate
Pgh 1	P-glycoprotein homolog 1
SAP	shrimp alkaline phosphatase
SDS-PAGE	sodium dodecyl sulphate–polyacrylamide gel electrophoresis
RNA	ribonuclease acid
TBP	TATA binding protein
TM	transmembrane helix
TMD	transmembrane domain
UTR	untranslated region

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1 Introduction

Acanthamoeba castellanii is an opportunistic human pathogen causing ocular infection called acanthamoeba keratitis and the life threatening granulomatous acanthamoeba encephalitis, which mainly effects immunocompromised patients. *A. castellanii* infections are rare, hence they are often neglected, even though they present a serious issue in human health. Therefore, it is necessary to pay more attention to its treatment. Although treatment options are available, they are in many cases ineffective, as a consequence of failure of unspecific chemotherapeutics and late diagnosis. The reason behind resistance of *A. castellanii* is not fully known, however, it is mainly attributed to the formation of resistant cysts.

The mechanism of resistance in trophozoites, a significant gap in current research, is the main premise of this thesis. There is a number of possible explanations for the emergence of resistance. A phenomenon described in various types of pathogens is the multi drug resistance (MDR), which is caused by cellular efflux pumps also known as ATP binding cassette (ABC) transporters. These transporters are responsible for the efflux of xenobiotics, which prohibits them from reaching an effective concentration inside the cell. Thorough research was conducted on ABC transporters' role in antibiotic resistance in bacteria, fungi and most importantly in cancer resistance to chemotherapy, however, no research is available in *A. castellanii*.

A novel ABC transporter (ACA1_352460) was recently discovered in *A. castellanii* in an analysis of the impact of oxidative stress. The aim of this thesis is the characterisation of this ABC transporter and its involvement in the drug resistance of *A. castellanii*. This could provide insight into the molecular mechanisms of *A. castellanii* resistance to chemotherapeutics and lay ground for better understanding of MDR. In addition, characterising the influence of the ABC transporter on the efficacy of treatment should contribute to the improvement of chemotherapeutics usage.

As molecular biology progresses it is important to implement knockout methods into *A. castellanii*. A knockout method utilizing homologous recombination was carried out in order to test the phenotypic influence of the ABC transporter. In addition, a recently published CRISPR Cas9 in *A. castellanii* was tested to achieve better efficacy and establish foundation for future research.

2 Review of literature

2.1 ATP-binding cassette transporters

The family of ATP-binding cassette (ABC) transporters is present in all supergroups of eukaryotes and in bacteria. Its primary function is to transport molecules across the lipid membrane causing hydrolysis of adenosine triphosphate (ATP). Substrates of the ABC transporters vary from organic molecules to metal ions and inorganic compounds. Thus, they are engaged in many cellular processes and have an abundant presence in cellular compartments (Liu, 2019). ABC transporters were first described in drug resistant ovary cells of a Chinese hamster. These cells exhibited lower permeation of various drugs and proteases (Juliano & Ling, 1976).

2.1.1 Structure and transport mechanism

The general structure of ABC transporters is highly conserved in all types of organisms and consists of four domains. Two of them are transmembrane domains (TMD) formed by six α-helices, and each TMD is associated with an intracellularly placed nucleotide binding domain (NBD) (Hyde et al., 1990) (Figure 1). The quaternary structures of ABC transporters differ mainly when it comes to symmetry, particularly the number and composition of transcript units that form them. The genes of one ABC transporter can be expressed from a single transcript unit, or its domains are fused and form different combinations of transcript units (Hyde et al., 1990; Xiong et al., 2015).

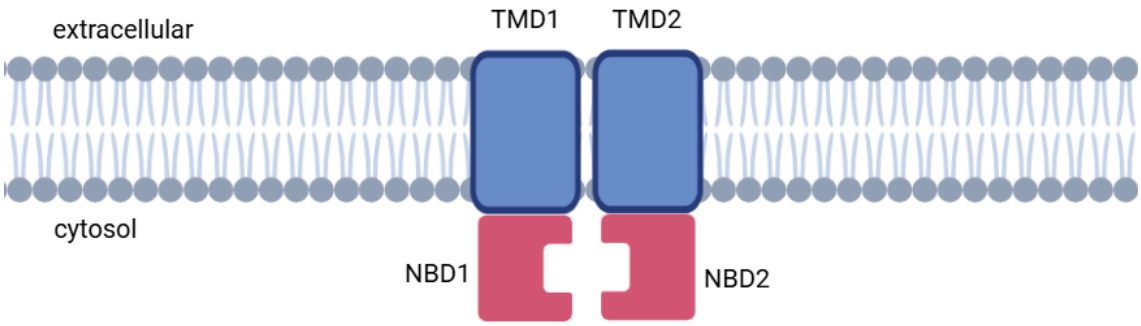

Figure 1. Schematic structure of ABC transporter located in the cytoplasmic membrane. The ABC transporters consist of two transmembrane domains (TMD) and two nucleotide binding domains (NBD). Created in BioRender.

Eight subfamilies of eukaryotic ABC transporters have been recognized based on their sequence similarity and quaternary structure. Subfamilies ABCA, ABCB, ABCC, ABCD, ABCG possess two TMDs and two NBDs and are involved in the transport of molecules from the cell. This group can further be divided into type 1 and type 2 exporters, which differ in the TMD structure. ABCE, ABCF, ABCH take part in cellular processes like translation or DNA repair, therefore, the TMDs are absent (Xiong et al., 2015).

In the presence of a ligand the transporter positions itself to an inward open state and the TMDs form a binding pocket for the ligand to enter. A change of conformation occurs upon binding of ATP, causing dimerization of the NBDs, which subsequently opens the pocket outwards. The ligand is released, and to restore the initial state of the transporter a hydrolysis of ATP follows (Pan & Aller, 2018; L. Wang et al., 2020) (Figure 2).

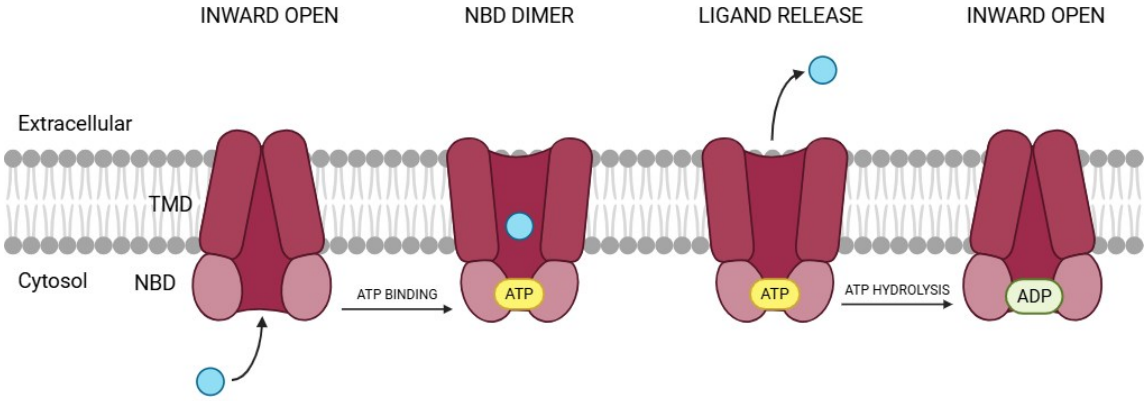

Figure 2. The cycle of ABC transporter. Efflux of the substrate (blue) is executed by conformational changes in the opening of ABC transporter. Before binding, the transporter faces inwards. The substrate binds into the pocket of two TMDs and upon binding of ATP the NBDs dimerize, and the transporter changes its conformation. Now facing outwards, the ligand is released, and ATP is hydrolysed, which results in the ABC transporter opening inwards. Abbreviation: TMD – transmembrane domain, NBD – nucleotide binding domain, ATP – adenosine triphosphate, ADP – adenosine diphosphate. Created in BioRender.

The interaction between the TMDs and NBDs is facilitated by coupling helices. One helix is extended from the TMD and placed onto the NBD, the interaction surface resembles a groove. The coupling helices are responsible for the coordination of movement of the TMDs and the NBDs and translocation of the substrate. Particularly, the change of conformation relies on ATPase activity, which is supported by the coupling helices (Furuta et al., 2014). The ATP binding site consists of multiple interacting chains (Figure 3). One of the interacting chains of the active site is the P loop or the Walker A motif. The P loop fold contains highly conserved residues, which bind the β-phosphate and orients the ATP into a position for hydrolysis (Kozlova et al., 2022; Oldham & Chen, 2011). The other chain is the A loop, which is necessary for stabilizing the interaction with ATP. It is in a proximity of the P loop and supports it via aromatic amino acids, thereby increasing affinity to ATP as well (De la Rosa & Nelson, 2011). The dimerization of the NBDs is facilitated by a signature LSGGQ motif, which is inserted into the catalytic pocket of the other NBD upon ATP binding (Kozlova et al., 2022; Oldham & Chen, 2011). After binding and orientation of ATP, it is most likely cleaved by a mechanism involving a glutamate residue as a key actor, this occurs in a Walker B motif catalytic site (Orelle et al., 2003). Additionally, the ATP hydrolysis is mediated by the D loop, which is also involved in the transport mechanism (Jones & George, 2012).

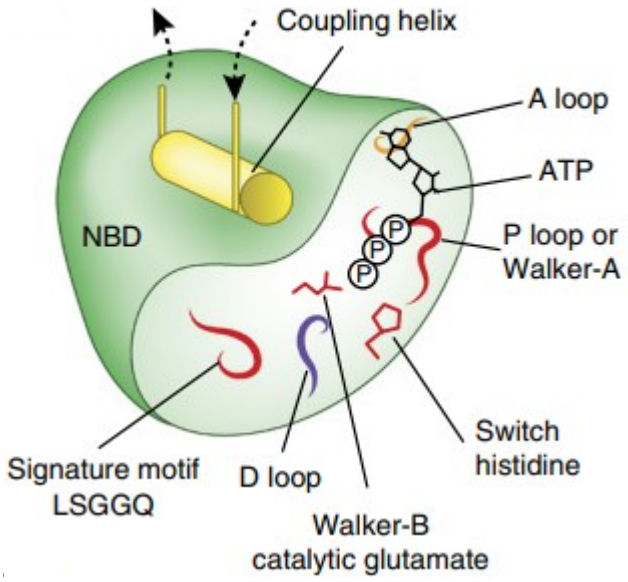

Figure 3. The nucleotide binding domain of an ABC transporter with highlighted ATP and its interaction partners (Locher, 2016).

Some ligands are able to stimulate hydrolysis of ATP and therefore facilitate a faster overturn, or in other words, save energy in low substrate concentration (Wang et al., 2020). Different interaction sites within the binding pocket are shaped depending on the character of the ligand. The size and hydrophobicity are being taken into consideration while determining its location within the binding pocket. To enable the transport, two transmembrane helices (TM) exhibit movement upon binding of the substrate and depending on the nature of the ligand different helices interact. It is noteworthy that the binding of xenobiotics utilizes unexpected helices duplets (Szewczyk et al., 2015). Furthermore, it was proposed that the ABC transporters operate with the induced-fit mechanism, which means they are constantly changing their opening position and creating new binding sites. Considering this, some drugs might be able to create a distinct binding site, and the ABC transporters could transport unlimited number of substrates (Esser et al., 2017; Szewczyk et al., 2015; L. Wang et al., 2020).

2.1.2 ABC type 2 transporters

From the family of ABC transporters two subfamilies, namely ABCA and ABCG, can be put into one group based on their structural similarity, forming ABC type 2 transporters. Like the type 1 ABC transporters, they are composed of two cytosolic NBDs and two transmembrane TMDs. However, due to their procaryotic descend, an extracellular extension of each TMD forms an extracellular domain (ECD), covering the TMD of the other half (Figure 4). On the other hand, unlike the type 1, the type 2 TMDs possess almost no cytosolic extension, bringing the NBD closer to the cytoplasmic membrane (Lee et al., 2016; Qian et al., 2017; Taylor et al., 2017). Furthermore, an outward facing conformation independent of ATP binding was described only in

the subfamily ABCA. In contrast, the ABCG NBDs do not interact unless ATP is bound (Qian et al., 2017). The TMDs are two separate domains that do not interchange helices with one another and fold independently. The limited interface of the TMDs is suitable for transport of hydrophobic substrates, due to their direct access to the lipid bilayer and residue composition of the binding pocket.

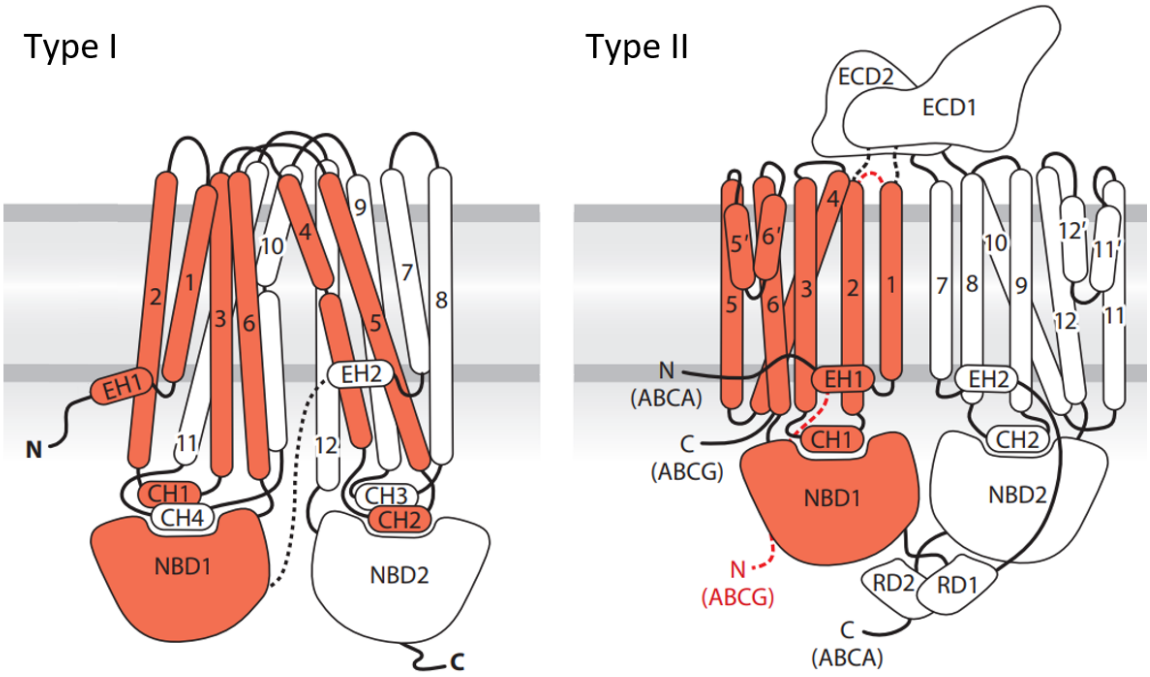

Figure 4. Comparison of structures of ABC type 1 and ABC type 2 transporters. Both types consist of two transmembrane domains, each is formed by six helices and two nucleotide binding domains (NBD). ABC transporters are to a certain extent half symmetrical (orange and white). In the ABC type 1 transporters two helices (4, 5) from one transmembrane domain are located in the other transmembrane and interact with the opposite NBD, and vice versa. In the ABC type 2 the transmembrane domains are divided into two discrete parts without any contact with one another. Furthermore, the ABC type 2 transporters possess two extracellular domains (ECD) located above the transmembrane domains (Alam & Locher, 2023).

The translocation of the substrates corresponds to the generic ABC transporter transport mechanism (Figure 2). The inward facing state changes upon ATP binding, closing the binding pocket by NBDs dimerization (Lee et al., 2016; Qian et al., 2017; Taylor et al., 2017). The outward facing cavity has been described to be more hydrophilic than the binding pocket, suggesting its adaptation for the release of a more hydrophobic substrate. The hydrolysis of ATP initiates a conformation, that reverts to the inward facing state (Taylor et al., 2017). One of the key characteristics of the ABC type 2 transporters is their ability to flip phospholipids in biological membranes from one layer to the other. This mechanism, based on loading the phospholipid bilayer, is crucial in cholesterol transport and formation of high-density lipoprotein vesicles (Quazi & Molday, 2013).

2.1.3 Human ABC transporters

Numerous ABC transporters are present in the human organism and often partake in essential biological processes. Defects of the ABC transporters cause diseases, which can interfere with everyday life and may even be life-threatening.

2.1.3.1 Cystic fibrosis transmembrane conductor

The ABC transporter cystic fibrosis transmembrane conductor (CFTR) is located in the cytoplasmic membrane of epithelial cells, where its primary function is to transport chloride and bicarbonate across the membrane. Mutations in the *CFTR* gene cause deficiency in mucus production on epithelium, leading to a chronic illness known as cystic fibrosis, which effects the respiratory tract. However, the CFTRs are present in plethora of tissues, for example in the pancreas, sweat glands or the digestive tract (Riordan et al., 1989). As a member of the ABC transporter protein family, the CFTR consists of two TMDs and two NBDs. Additionally, it was found it contains another, highly positively charged cytoplasmic domain, the R domain, which is unique to the CFTR. It interacts with the NBDs to mediate phosphorylation and subsequent opening of the ion channel. The R domain itself possesses sites for the protein kinase A (Baker et al., 2007; Riordan et al., 1989).

The cystic fibrosis is an autosomal recessive disorder with approximately 200 known mutations in the CFTR, with the most prevalent mutation in Phe508 accounts for nearly 70 % of cases (Csanády et al., 2019; McKone et al., 2003; Riordan et al., 198). The consequences of the gene mutation can manifest on several levels: protein production, processing and localisation or defective function (McKone et al., 2003).

Therapeutics for cystic fibrosis have developed in the recent years from only treating the symptoms to targeting the particular deficiencies of the CFTR ion channel. A group of potentiators enhances the transport of ions through the transporter via stimulation of the open state, hence prolonging the duration of the opening (Yeh et al., 2017). Two drugs, VX-770 and GLPG1837, with the similar mechanisms and binding sites, are in use, however, efficacy of these drugs varies in different mutations (Yeh et al., 2017, 2019).

2.1.3.2 ABCA4

ABCA4 is a protein localised in the cytoplasmic membrane of a photoreceptor disc, and its primary function is to export toxic compounds (Molday et al., 1987). Transduction of signal in rods produces potentially toxic agents from retinal pigment, which then needs to be extruded from the cell (Kim et al., 2007). Defects in ABCA4 lead to macular dystrophies and vision impairment, which are also known as Stargardt disease (Allikmets et al., 1997). It shares the structural properties of the standard ABC transporter structure, which consists of a half symmetrical structure formed by two TMDs and two NBDs. Its distinct feature are two exocytoplasmic

domains expanded from the first loop, one in each of the two TMDs, which form a disulfide bond between the two exocytoplasmic domains (Bungert et al., 2001).

A promising approach to genetic diseases is gene therapy, which targets the primary cause of the pathology. A non-viral nanoparticle therapy has been proposed, where the nanoparticle delivers a DNA plasmid with a rhodopsin promotor to achieve expression of ABCA4 in rods (Sun et al., 2020). The application of this particular therapy showed a 40 % improvement of processed retinal pigment accumulation after 6 months, as well as a significant rise of mRNA and protein levels of ABCA4 (Sun et al., 2024).

There are currently drugs in clinical trial for Stargardt disease, although none have been approved by the FDA yet. Pharmaceutics inhibit the accumulation of toxic compounds from the visual cycle, either via slowing the visual cycle, therefore, lowering the production of toxic agents, or via inhibiting the formation of toxic agents from their precursor (MacDonald & Sieving, 2018; Piccardi et al., 2019; Radu et al., 2003).

2.1.4 ABC transporters involved in drug resistance

One of the biggest challenges in drug research and treatment is a quick development of resistance, which affects a wide range of diseases caused by parasites, bacteria or cancer. The failure of treatment has many forms and is often facilitated by ABC transporters, also known as the agents in MDR development (Magiorakos et al., 2012). Due to their adaptability to new ligands, the ABC transporters possess the ability to export diverse ligands, including pharmaceutics, from the cell, and therefore significantly decreasing their effectiveness (Juliano & Ling, 1976; Nosol et al., 2020). The majority of research on MDR is being pursued in connection with cancer treatment, however, it provides an important insight into how drug resistance emerges, which can bring clarity into drug resistance issues in parasitic diseases.

The most profoundly studied ABC transporter and the first to be discovered is P-glycoprotein, which is physiologically present in cells. In cancer cells certain phenotypes promote MDR, resulting in hindrance of treatment caused by overexpression of ABC transporters or by changes in nucleotide sequence (R. C. Wang et al., 2017). The structure of P-glycoprotein is simple, it consists of two TMDs and two NBDs (Juliano & Ling, 1976). Several mutations were proposed to partake in the rate of efflux, mostly regarding cancer treatment. One example of gained MDR by mutation in NBDs is the change of serine to asparagine in the position 400, which exhibits a higher resilience to anticancer drugs and alters the permeability of epithelial cells (Woodahl et al., 2009). However, certain mutation can have the opposite effect depending on the treatment. For example, Björn et al. (2018) described a case, where a single nucleotide polymorphism in P-glycoprotein exhibited phenotypes opposite to expectations, emphasizing the importance of individual treatment and attention to the genotype of the patient.

2.1.4.1 Parasites

The MDR phenotype was also discovered within the apicomplexan group. A protein homologous to P-glycoprotein was discovered and it was presumed to have a function in the resistance to chloroquine in *Plasmodium falciparum.* This protein named P-glycoprotein homolog 1 (Pgh1) is localised in the membrane of the digestive vacuole and facilitates transport from cytosol to the digestive vacuole (Cowman et al., 1991). The Pgh1 is not the primary cause of chloroquine resistance, however, it is utilized in the resistance to other chemotherapeutics. Overexpression or higher number of gene copies of Pgh1 correlated with resistance to mefloquine, artemisinin and halofantrine (Elandaloussi et al., 2006; Nishiyama et al., 2004; Sidhu et al., 2006; Yan et al., 2025). Interestingly, overexpression of Pgh1 resulted in more susceptibility to chloroquine, however, that coincides with the fact that chloroquine is impactful only in the digestive vacuole, and mutations in Pgh1 show resistance to chloroquine (Nishiyama et al., 2004; Venkatesan et al., 2014). Overall, Pgh1 is responsible for transport of chloroquine into the digestive vacuole, where it is toxic, thus increasing the susceptibility of *P. falciparum* to chloroquine. However, drugs like artemisinin, mefloquine and halofantrine, which are degraded in the digestive vacuole, are less effective in the presence of Pgh1.

P-glycoprotein is also proposed as one of the potential mechanisms for ivermectin resistance in nematodes, namely in the sheep nematode *Haemonchus contorsus* (M. Xu et al., 1998). Studies of the *H. contorsus* P-glycoprotein yielded diverse results in different life stages and homologs. *In vivo* studies on adult stages found a parallel with overexpression of P-glycoprotein and resistance to ivermectin. Although, these results were not consistent enough and suggested various mechanisms of resistance (Maté et al., 2018). However, a clear correlation with ivermectin resistance was observed in the P-glycoprotein 16 in adults and L3 stages, where the P-glycoprotein 16 showed high levels of expression and consequently was proposed as a marker for ivermectin resistance in *H. contorsus* (Reyes-Guerrero et al., 2020). Generally, ABC transporters promote various resistances to chemotherapeutics in helminths and are not restricted to nematodes (Kasinathan et al., 2010, 2014; Sanchez et al., 2017).

ABC transporters were recognized as a potent agent in resistance to multiple leishmaniasis treatments. *Leishmania* was recognized to possess a mechanism for detoxification of antimony, utilizing intracellular ABC transporters, while treated by antimonial compounds. This system is, however, dependent on other factors. It only sequesters metal-thiol conjugates, therefore, the glutathione detoxification pathways are necessary (Campos-Salinas et al., 2013; Perea et al., 2018). Higher expression of this transporter was detected in amastigote and promastigote life stages (El Fadili et al., 2005; Légaré et al., 2001). In addition, a set of ABC transporters has been identified as partaking in decreased susceptibility to treatment with amphotericin B or miltefosine (Castanys-Muñoz et al., 2008; Jariyapan et al., 2025; Obonaga et al., 2014).

2.1.4.2 Inhibitors of ABC transporters

One of the solutions to the occurrence of MDR and continuous failures of treatment is the development of MDR transporter inhibitors. These inhibitors were developed in response to the failure of cancer treatment, however, it could serve as potential treatment option for parasitic diseases. Several inhibitors have been proposed, but despite their success in increasing efficacy of treatment, the drugs had many side effects and were soon rejected and replaced by the third generation of MDR inhibitors (Björn et al., 2018; Nosol et al., 2020; Woodahl et al., 2009). Currently used P-glycoprotein inhibitors, including vinblastine/vincristine, zosuquidar, tariquidar and elacridar, bind into the binding pocket (Figure 5). All of them show a significant decrease of xenobiotics efflux in cells overexpressing P-glycoprotein. In the cryo-electron microscopy analysis conducted by Nosol et al., 2020, the binding of small molecules was observed and revealed two mechanisms of these four inhibitors. Vincristine binds and alters the binding pocket through the formation of transient bonds, which lower the ATPase activity by blocking the hydrolysis of ATP. Elicridar and tariquidar were described to possess different mechanisms of inhibition, each binding two molecules inside the binding pocket. The first molecule behaves as a substrate, adopting an U-shaped conformation (Figure 5, middle panel in yellow and aqua) and the second molecule inhibits the transporter, adjusting itself to an L-shaped conformation, and reaches outside the binding pocket (Figure 5, middle panel in orange and green). On the contrary to vincristine, the ATPase activity is still present due to the flexibility of the NBDs, but the transport of molecules is inhibited. This further confirms the adaptibility of the binding pocket to different subsrates (Alam et al., 2019; Nosol et al., 2020). Furthemore, the rates of the ATPase increase upon binding of some substrates as a result of steric changes and the tightening of the two halves (Alam et al., 2019).

Even though the majority of research regarding MDR inhibitors is for cancer treatment, it is of great significance for resolving cases of parasitic drug resistance, due to the ability of parasites to quickly develop resistance to treatment which is often facilitated by ABC transporters. To date there is a very limited knowledge about ABC transporters and MDR in *A. castellanii*, thus further research might reveal, how ABC transporters are involved in treatment failure of acanthamoeba infections.

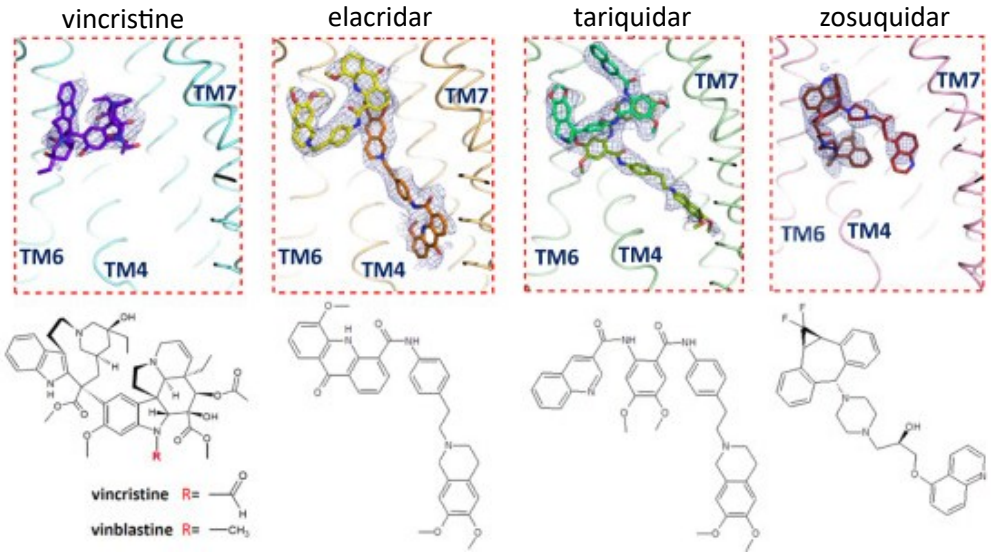

Figure 5. Binding of different inhibitors into the binding pocket of ABC transporters (red rectangles). In the lower half of the figure are the molecular structures of four inhibitors – vincristine, elacridar, tariquidar, zosuquidar. In the red rectangles are the conformations of the inhibitors inside the binding pocket with highlighted interacting helices (TM4, TM6, TM7) (Nosol et al., 2020).

2.2 *Acanthamoeba castellanii*

A. castellanii is an amphizoic cosmopolitan amoeba found in aqueous environment and soil from the group Discosea, Amoebozoa (Tawfeek et al., 2016). It possesses a life cycle with two stages, a trophozoite and a cyst (Figure 6). The trophozoite is an amoebic metabolically active stage, which forms spine-like pseudopodia known as acanthopods. The main characteristic of amoebic cells is phagocytosis, which is a feeding strategy, where the particle is engulfed inside the cell and digested. *A. castellanii* is naturally a predator, their scraping of bacterial communities is essential for the ecosystem to maintain a stable state (Rodríguez-Zaragoza, 1994). The dormant cyst forms in unfavourable conditions and has a layered wall resembling a rounded star. The cyst wall consists of the endocyst, which is smooth on the surface and round, and the ectocyst, which is thicker with multiple bulges on the outer surface (Bowers & Korn, 1969). The main component of the cyst wall is cellulose, which is present in both endocyst and ectocyst (Dudley et al., 2009; Garajová et al., 2019; Tomlinson & Jones, 1962). Moreover, it contains other saccharides and proteins like lectins, which bind well to cellulose (Magistrado-Coxen et al., 2019). The cyst is resistant to some disinfecting agents and its formation can cause ineffectiveness of chemotherapeutics (Khan, 2006).

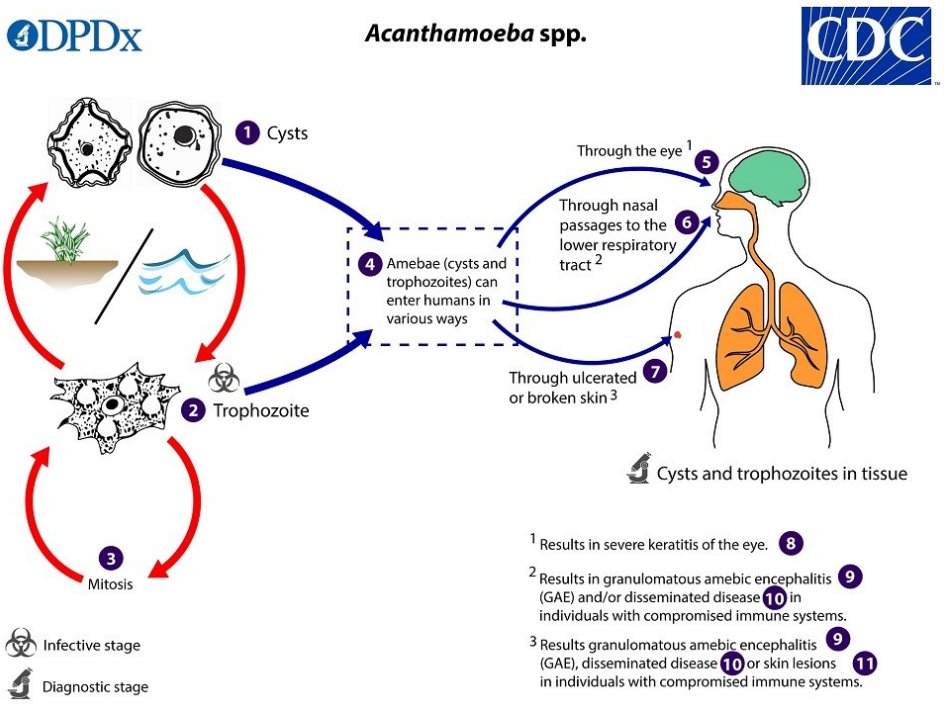

Figure 6. The life cycle of *A. castellanii*. Two stages: trophozoite and dormant cyst. Each stage can be found as a free-living organism in nature or opportunistically as a pathogen (CDC, 2024).

Due to its dual lifestyle ability, it is an opportunistic parasite causing life threatening diseases mainly in immunocompromised patients (Martinez, 1982; Wiley et al., 1987). Namely acanthamoeba keratitis (AK), a corneal infection, causes permanent sight damage, but does not spread to other organs (Naginton et al., 1974). Acanthamoeba keratitis is a rare disease with approximately 20 000 cases per year globally, and the growth of this number correlates with the increase of usage of contact lenses (Zhang et al., 2023). The infection is prominent among contact lens users, because of contamination of the contact lenses with cysts either from water or by unsanitary manipulation (Lindsay et al., 2007). Another way of infection is due to eye trauma, which makes an easy gateway for the infection. *A. castellanii* adheres to the surface of the eye and invades the eye through the collagen fibres and progresses further into the stroma of the cornea. In some cases a circular infiltrate, called radial keratoneuritis, is formed around the iris (Omaña-Molina et al., 2014).

Secondly, *A. castellanii* causes granulomatous amoebic encephalitis (GAE), which leads to chronic inflammation of the central nervous system and often results in death (Fowler & Carter, 1965). Amoeba usually invading the host through skin injuries or lungs and disseminates by the bloodstream to the brain. The trophozoite crosses the haematoencephalic barrier, then invades the brain, and that leads to inflammation of the tissue (Dewan Duggal et al., 2017). GAE progresses slowly with unspecific symptoms like headache, nausea or fever. Within months it becomes more severe with neurological symptoms and in 90 % is fatal (Aparicio et al., 2021; Lau et al., 2021; Matsui et al., 2018). In addition, there have been reports of GAE in immunocompetent patients

caused by *A. castellanii*, however, these infections are very rare (Aparicio et al., 2021; Gelman et al., 2001; J. Liu et al., 2023).

Alongside with the neurological symptoms of GAE, immunocompromised patients can experience skin infection after the dissemination of the amoebae from the brain or direct infection of the skin. The skin is inflamed with subcutaneous nodules that increase in size. After four to six months since the first symptoms the nodule ruptures and forms an ulcerous. The skin lesions in some cases remain closed (Tan et al., 1993) and heal after ulceration (Gullett et al., 1979). In other cases, nonhealing lesions are formed (Jiang et al., 2024).

Furthermore, *A. castellanii* contains a wide range of symbiotic bacteria and viruses (Iovieno et al., 2010; Scola et al., 2003; Seltzner et al., 2021). The endosymbionts developed the ability to persist phagocytosis in amoebae by altering their digestion and thus can survive inside the cell in various organelles (Hay et al., 2023). Bacteria become more persistent in *A. castellanii* and are mutualists, however, their presence can have the opposite, cytopathic, effect on their host (Buse et al., 2016). The relationship with symbiotic bacteria, on the other hand, can be a necessity. For example, the presence of bacterial endosymbionts helps the development and enhances the severity of acanthamoeba keratitis (Nakagawa et al., 2015). In addition, transmission of bacteria to humans through *A. castellanii* has been described. Cases of *Legionella pneumophila,* an opportunistic pathogen and an agent in pneumonia also known as Legionnaires' disease, were reported to be transmitted by *A. castellanii* and contracted via contaminated water supplies and air conditioning (Edagawa et al., 2008; Newton et al., 2010; Yamaguchi et al., 2017).

2.2.1 Virulence of *A. castellanii*

A. castellanii virulence manifests mainly upon direct contact with the host cells via adhesion and phagocytosis. However, amoebae possess mechanisms to act cytotoxic without the requirement of direct contact with the host cells utilizing exosomes and secretion of proteases into the medium. In addition, the formation of the cyst further advances the pathogenic effect of *A. castellanii*, due to its resistance to the immune system and chemotherapeutics. These factors contribute to complex pathogenicity in the accidental human host.

Secretion of virulence factors is an important element in the cytotoxicity of *A. castellanii* upon infection. The secretome, mostly consisting of serine, cysteine proteases and metalloproteases, can alone be responsible for lysis of the host cells, however, the induction of apoptosis solely by secretome showed contradictory results. Furthermore, the secreted proteases compromise cell viability by disrupting the host cell actin cytoskeleton, which might enable easier adhesion and invasion of *A. castellanii* (Alhazmi et al., 2024). Additionally, the secreted proteases disrupt host cell junctions, thus promoting the lysis of the affected cells and their release to the medium. This was observed by Huang et al. (2017) as a strategy to phagocyte the host cells. The relationship of *A. castellanii* and environmental microbiome is known to enhance pathogenicity. One of the

processes present in their interaction is the horizontal gene transfer (Bertelli & Greub, 2012; Nakagawa et al., 2015). *A. castellanii* endosymbionts enrich its genome in size with functional genes present in metabolic pathways and cellular signalling. This influences its virulence and pathogenicity among *A. castellanii* strains. Multiple proteases families were identified to be acquired via horizontal gene transfer and potentially involved in the pathogenic processes of *A. castellanii* (Gu et al., 2022).

Adhesion to the host cells is one of the first actions defining *A. castellanii* virulence. The amoebae recognise their pray or their accidental human hosts cells utilizing adhesins, mostly mannose-binding protein and laminin-binding protein (Corsaro, 2022). The adhesion is crucial especially in AK, to invade the corneal epithelium (Garate et al., 2006). Recently, a study examined an ectophosphatase that can use extracellular substrates without internalization in connection with adhesion to the host cell. Its presumed function is facilitating adhesion upon contact with mannose through a mannose-binding protein (Carvalho-Kelly et al., 2023). It was speculated that the ectophosphatase may have a role in immune evasion, due to its ability to hydrolyse ATP (de Souza-Maciel et al., 2024).

A characteristic activity of amoebae is phagocytosis, a process of engulfment of particles or bacteria from the environment, which is essential in pathogenesis of *A. castellanii* (Alsam et al., 2005). A well-known, although poorly described structure in *A. castellanii* is the amoebastome, a bowl-shaped structure on the surface used for phagocytosis. The amoebostome was described in the presence of mammalian cells and used for phagocyting of detached cells (Omaña-Molina et al., 2004).

2.2.2 Treatment of *A. castellanii* infections

Treatment of diseases caused by *A. castellanii* is often unsuccessful due to the nature of infection, formation of cysts and false diagnosis (Campolo et al., 2022). Chemotherapeutic strategies comprise of antimicrobial and antifungal agents. Corneal infections are treated with antiseptic solutions in the form of eye drops with the active agents being biguanides or chlorhexidine combined with diamidines (CDC, 2025). Biguanides have shown toxic effects for trophozoites and cysts and have thus been classified as the most competent treatment (Büchele et al., 2023; Noradilah et al., 2010). To enhance the amoebicidal effect, the treatment can be combined with antifungal drugs, which are toxic to trophozoites and cysts (Shing et al., 2020).

The mechanism of function of these drugs is often unresolved, however, there are studies conducted to clarify its amoebicidal effect. Moreover, some *A. castellanii* strains are resistant to polyhexamethylene biguanide and several proteins have been identified to promote its resistance, mostly via encystation. In order to decrease the resistance caused by encystation, the inhibition of P-ATPase, which is an ion channel located in the endoplasmic reticulum, was studied. A compound ouabain, an ATPase inhibitor, was able to decrease the rate of encystation

via inhibiting the ATPase, and thus lower the resistance to polyhexamethylene biguanide in *A. castellanii* (Shih et al., 2024). Cytochrome P450 monooxygenase might be responsible for the detoxification of polyhexamethylene biguanide and its overexpression averted encystation. Therefore, this enzyme induces a different mechanism to overcome toxic agents (Huang et al., 2021).

Formation of cysts is a deciding factor in the development of AK and simultaneously the cause of resistance for drugs primary targeting trophozoites like chlorhexidine. In a recent study, the use of lactase was proposed to inhibit cyst formation and therefore enhance the effect of chlorhexidine. Lactase is an enzyme responsible for the cleavage of carbohydrates, which are present in the cyst wall and are important for its formation. Overall, lactase could be considered as a candidate to increase the efficacy of treatment and disinfecting solutions (Simau et al., 2024). Furthermore, the trophozoite stage possesses a disulfideisomerase involved in resistance to polyhexamethylene biguanide. This enzyme was highly expressed in resistant isolates trophozoite stage and induced cyst independent resistance (F.-C. Huang et al., 2016). Identification of trophozoite stage factors in drug resistance gives an important insight into development of future treatment and targeting particular agents in drug resistance.

For non-keratitis infections the spectrum of drugs is far more diverse consisting mostly of oral drugs, except for pentamidine, which is administered intravenously. Although, pentamidine showed toxicity towards the patient and does not cross the blood-brain barrier sufficiently. The drugs most used for GAE are sulfadiazine and flucytosine (CDC, 2025). It was proposed that miltefosine, a drug developed against cancer but nowadays used to treat leishmaniasis, has an amoebicidal effect as well and shows potential in treating GAE and AK (Chan et al., 2022; Hirabayashi et al., 2019).

Very few studies regarding the molecular mechanism of *A. castellanii* resistance to GAE treatment are available, however, the drug flucytosine, also used to treat candidiasis, lost its effect after resistance have emerged in some *Candida* strains (Delma et al., 2025). *Candida* spp. possesses a variety of MDR transporters involved in resistance to antimycotics, which are often used or considered for treating *A. castellanii* infections. This could draw a parallel in resistance development to treatment and potentially increase the treatment efficacy (Abou-Chakra et al., 2025; Rajesh-Khanna et al., 2025; Taravaud et al., 2017).

3 Aims of this thesis

1. Characterise the newly discovered ABC transporter in *A. castellanii.*

2. Describe the function of the ABC transporter in drug resistance of *A. castellanii*.

3. Develop and implement the knockout method.

4. Prepare an ABC transporter knockout and characterize its phenotype.

4 Methods

4.1 Polymerase chain reaction (PCR)

Each PCR reaction was performed in accordance with the manufacturer's instructions. The primer annealing temperature was optimized using the NEB Tm calculator (https://tmcalculator.neb.com/#!/main). The PCR master mixes are listed in Table 1.

Table 1. Master mixes used for PCR.

PCR master mix	Manual
Q5 High Fidelity 2X Master Mix (New England Biolabs)	https://www.neb.com/en/protocols/2012/12/07/protocol-for-q5-high-fidelity-2x-master-mix-m0492
2X Phusion Green Hot Start II High Fidelity PCR Master Mix (Thermo Scientific)	https://www.thermofisher.com/order/catalog/product/F566S
SapphireAmp fast PCR – hot-start master mix (TaKaRa)	https://www.takarabio.com/products/pcr/pcr-master-mixes/dye-added/sapphireamp-fast-premix

4.2 Ligation reactions

T4 ligase (Thermo Scientific) and 10X T4 DNA Ligase Buffer (Thermo Scientific) were used in all ligation reactions. pGEM-T Easy (Promega), suitable for TA cloning, and CloneJET PCR Cloning vector (Thermo Scientific), suitable for blunt end cloning, were used as cloning vectors. Vectors pET-42b and pET-31c were used to express recombinant proteins. DNA concentrations were adjusted in accordance with the manufacturer's instructions.

4.3 Restriction digest

Restriction digest reactions were carried out utilizing FastDigest (Thermo Scientific) products. The reactions were mixed in accordance with the manufacturer's manual (https://assets.thermofisher.com/TFS-Assets/BID/Reference-Materials/fastdigest-restriction-enzymes-labaid.pdf), apart from the incubation time, which was extended to 1 hour.

4.4 Gibson cloning

The Gibson cloning master mix was prepared as stated in Table 2 and Table 3. It was then divided into aliquots of 10 µl, which were thereafter used to assemble the reaction as stated in Table 4.

Table 2. Components of 5x ISO buffer used for Gibson cloning reactions.

5x ISO buffer	
Tris-HCl (pH 7.5)	0.5 M
$MgCl_2$	50 mM
dNTPs	4 mM
DTT	50 mM
PEG-800	4 %
NAD	5 mM

Table 3. Components of the Gibson cloning master mix (1.2 ml). All individual reactions are stored in aliquots of 10 µl at -20 °C.

Gibson cloning master mix	
5X ISO buffer	320 µl
10 U/µl T5 exonuclease	0.64 µl
2 U/µl Phusion polymerase (Thermo Scientific)	20 µl
40 U/µl Taq ligase (New England Biolabs)	160 µl
Nuclease free water	700 µl

Table 4. Components of the Gibson cloning reaction.

Gibson cloning reaction	
Master mix	10 µl
Linearised vector	200 ng
Insert	1:1 ratio insert to vector
Water	up to 20 µl

4.5 Agarose gel electrophoresis

As for gel separation of DNA fragments, a 1 % agarose (Sigma-Aldrich) gel was prepared using TAE buffer as diluent. To visualise the DNA, 50 µl of Sybr Safe (Thermo Scientific) were added to 10 ml of the agarose gel. The Sybr Safe is a type of dye, that binds to DNA and emits light when exposed to UV light. GeneRuler DNA Ladder Mix (Thermo Scientific) was used as standard.

4.6 DNA isolation from agarose gel

After the gel separation, suitable bends were cut from the gel, and the DNA was extracted using the E.Z.N.A. Gel Extraction Kit (Omega Bio-tek).

4.7 Nucleic acids concentration measurements

All the DNA/RNA samples were measured using a NanoDrop 2000 spectrophotometer (Thermo Scientific).

4.8 Transformation of *Escherichia coli*

A TOP10 strain of competent *E. coli* cells were incubated with 10 µl of ligation reaction or with 150 ng of plasmid for 20 minutes on ice. The cell membrane was permeabilized by heat shock at 42 °C for 30 seconds and immediately placed on ice for 2 minutes. Pre-warmed SOC medium (20 g/l tryptone, 5 g/l yeast extract, 10 mM NaCl, 2.5 mM KCl) was added to the tube containing the bacteria and placed on a horizontal shaker (220 rpm) at 37 °C for one hour. Prior to plating, the transformed cells were supplemented with antibiotics – the type of the antibiotic was determined, in every case, based on the plasmid resistance (Table 5). For the blue and white selection, 40 µl of X-Gal were added on an LB agar plate (20 g/l LB broth (Lennox), 12 % agarose (Sigma-Aldrich)). The plating was performed under semi-sterile conditions under a burner, and after the plate was dry, it was kept at 37 °C overnight.

Table 5. Concentrations of the antibiotics.

Antibiotic	Concentration
Ampicillin	100 µg/ml
Kanamycin	50 µg/ml

4.9 Plasmid isolation from *E. coli*

One bacterial clone from the LB agar plate was inoculated into 5 ml of LB liquid medium (20 g/l LB broth (Lennox)), which was supplemented with antibiotics as stated in Table 5, and incubated at 37 °C overnight. The grown culture was then pelleted, and the isolation process continued in accordance with the E.Z.N.A. Plasmid DNA Mini Kit (Omega Bio-tek) protocol.

4.10 SDS-PAGE protein electrophoresis

The samples in the form of a pellet were resuspended in a 1X sample loading buffer (50 mM TRIS base, 10 % glycerol, 2 % sodium dodecyl sulphate (SDS), 5 % β-merkaptoetanol, 0.002 % bromfenol blue (Serva)). The liquid samples were mixed with a 5X sample loading buffer (50 mM TRIS base, 10 % glycerol, 5 % sodium dodecyl sulphate (SDS), 5 % β-merkaptoetanol, 0.002 % bromfenol blue (Serva)). After that, all the samples were placed on a heating block for 5 minutes at 95 °C to denature all proteins.

All the experiments were carried out using 0.75 mm homogenous gels (5 % polyacrylamide stacking gel, 12 % polyacrylamide separating gel), which were prepared with a Mini-PROTEAN System (Bio-Rad). PageRuler Plus Prestained Protein Ladder (Thermo Scientific) was used as standard. The protein separation was carried out at a voltage of 100 V. In order to visualise all the separated proteins, the gels were stained with Coomassie Brilliant Blue (225 ml dH_2O, 50 ml

acetic acid 1 M, 225 ml methanol, 250 mg Coomassie Brilliant Blue R-250 (Serva)) for 1 hour at room temperature and subsequently destained with destain solution (600 ml dH_2O, 100 ml acetic acid 1 M, 250 ml methanol) for 30 minutes at room temperature a total of three times.

4.11 Immunoblotting

The proteins separated by the SDS-PAGE electrophoresis were transferred on a nitrocellulose membrane using a 70 mA current for 75 minutes, and a blotting buffer (100 ml 10X TGS (BioRad), 200 ml methanol, 700 ml dH_2O) was used as medium. The apparatus was assembled as followed: (from bottom to top) filter paper, nitrocellulose membrane (46 cm^2), polyacrylamide gel, filter paper. All the components were soaked in blotting buffer before the apparatus was put together. To confirm proper migration of proteins, the membrane was stained with Ponceau S (0.5 g/100 ml Ponceau S (Merck), 0.1 % acetic acid) and washed with dH_2O after the run was completed.

The membrane was blocked in 5 % nonfat-dried milk (in PBS) with 0.05 % Tween 20 (Sigma-Aldrich), hence forth referred to as blocking buffer, for 1 hour at room temperature, or overnight at 4 °C. The primary antibodies were diluted as stated in Table 6. The incubation time varied due to antibody specificity. After the incubation, the membrane was washed in blocking buffer for 10 minutes at room temperature a total of three times. The secondary antibodies conjugated with horseradish peroxidase were diluted 1:2000 and incubated with the membrane for 1 hour at room temperature (Table 6). The membrane was afterwards washed two times in blocking buffer for 10 minutes at room temperature and two times in PBS for 5 minutes at room temperature.

Table 6. List of antibodies used for western blotting. Ab - antibody

	Antibody	Dilution	Incubation conditions
Primary ab	Monoclonal mouse ab against His-tag	1:1000	1 hour, room temperature
	Polyclonal rat ab against ABC transporter	1:100	Overnight, 4 °C
Secondary ab	Ab against primary mouse	1:2000	1 hour, room temperature
	Ab against primary rat	1:2000	1 hour, room temperature

Horseradish peroxidase substrate (Sigma-Aldrich) was added onto the membrane directly before visualisation. Chemiluminescence of the horseradish peroxidase was visualised using Amersham Imager 600 (General Electric).

4.12 Cultivation of *A. castellanii*

A. castellanii genotype T4 was obtained from a clinical case of acanthamoeba keratitis (Hernández-Martínez et al., 2019) and maintained in an axenic culture. The cells were cultivated in an aerobic 25 cm^2 flask containing 10 ml of PYG media (Table 7) at 37 °C.

Table 7. Components of PYG medium.

Chemical	Volume/weight
Yeast extract (Oxoid)	7.5 g
Proteose peptone (Oxoid)	7.5 g
Glucose (Carl Roth)	15 g
KH_2PO_4 (Sigma)	0.272 g
$MgSO_4$ $7H_2O$ (1 M)	1 ml
$CaCl_2$ (1 M)	50 µl
Thiamine (1 µg/ml)	1 ml
Biotin (0.2 µg/ml)	800 µl
B12 (1 ng/ml)	10 µl
dH_20	up to 1 l

4.13 Preparation of polyclonal ABC transporter antibodies

The polyclonal rat antibodies were acquired by immunising a rat with a recombinant protein. The protein was chosen based on proteomic data from Ženíšková et al., (2025), because of its major upregulation in stress conditions.

4.13.1 Preparation of recombinant antigen in *E. coli*

The ABC transporter is a membrane protein with multiple transmembrane domains. Therefore, only a partial sequence, preferably a cytoplasmatic domain, must be chosen as the antigen. The ABC transporter sequence was downloaded from AmoebaDB and analysed in the Geneious Prime software. The antigen was synthesised using two approaches, that are outlined on Figure 7. The figure shows only those steps of the protocols, in which they differ. Two sets of primers were used and are stated in Table 8. The set of primers ABC1 corresponds to panel A on Figure 7 and the set of primers ABC2 corresponds to panel B on Figure 7.

Table 8. Sets of primers used for amplification of the ABC transporter and for antigen synthesis. In bold – restriction site, ▼- restriction cleavage

Primers for ABC transporter antigen	
ABC1 Ndel forward	ACA**CAT▼ATG**CTCAACATCGTGGCCAAGGT
ABC1 BamHI reverse	ACA**GGA▼TCC**GATCGCTGGACGAAAGCTTG
ABC2 Ndel forward	ACA**CAT▼ATG**AACTGCAGCTCTTGCACGAT
ABC2 BamHI reverse	ACA**GGA▼TCC**TTGAGCTGGA CGTAGAACGG

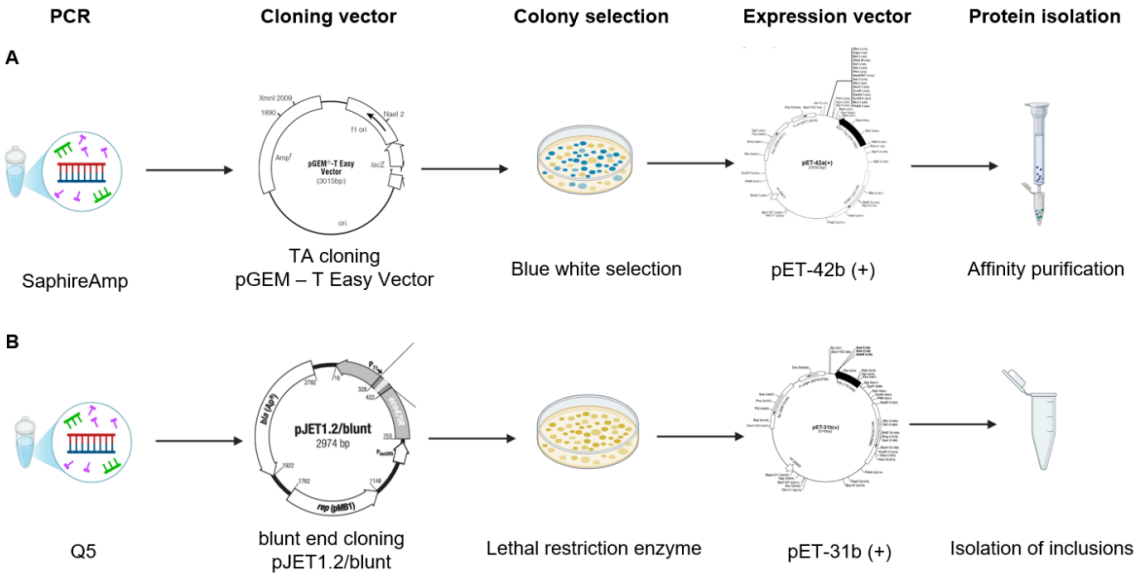

Figure 7. Schematic protocol for preparation of the ABC transporter antigen with two approaches (A and B), showing only those steps, in which they differ. Panel A: a polymerase that adds an adenosine at the end of the amplicon was used to allow TA cloning to the pGEM cloning vector (Sigma Aldrich). This vector contains lacZ operon, which enables blue and white selection. The antigen gene was subsequently isolated and cloned into an expression vector. The pET-42b (+) vector was used as the expression vector, and because it contained a polyhistidine tag (6x His), it was possible to perform purification of the protein using affinity chromatography. Panel B: a polymerase that leaves blunt ends was used for PCR, and the amplicon was cloned into pJET (Sigma Aldrich), a vector, that forms a lethal restriction enzyme, when ligated without insert. The pET-31b vector was used as the expression vector, in order to collect the protein from inclusion bodies. Created in BioRender

4.13.2 Preparation of expression vector

As a first step, total RNA was isolated from wild type strain of *A. castellanii* using the High Pure RNA Isolation Kit (Roche), which was then reverse transcribed into cDNA using the SuperScript III Reverse Transcriptase (Thermo Scientific). The sequence of interest was amplified by PCR using cDNA as template. The purified PCR product was ligated into cloning vectors (pGEM-T Easy, pJET 1.2/blunt). The ligation reaction was transformed into TOP10 *E. coli* strain and plated with 100 µg/ml of ampicillin. Selected colonies were further tested by PCR using the 2x Phusion Green Hot Start II High Fidelity PCR Master Mix (Thermo Scientific). The positive colonies were subsequently inoculated in 5 ml of LB media containing 100 µg/ml of ampicillin and cultivated on a shaker overnight at 37 °C. The plasmids were isolated from the bacterial cultures using the E.Z.N.A Plasmid DNA Mini Kit (Omega Bio-tek).

The insert was cleaved from the isolated plasmids using restriction enzymes (NdeI, BamHI). The cleaved insert was ligated into the expression vectors and subsequently transformed into *E. coli* strain BL21 and plated with 100 µg/ml of ampicillin (pET-31) or 50 µg/ml of kanamycin (pET-42b). The colonies were tested and cultivated as stated above.

4.13.3 Expression in *E. coli* BL21

The positive clones were inoculated into 5 ml of LB medium with 100 µg/ml antibiotics and cultivated overnight on a shaker at 37 °C. To test, whether the induction of gene expression of our protein of interest is functional, the following protocol was first carried out in a smaller volume. 200 µl from the grown bacterial culture were inoculated into 10 ml of fresh LB medium and kept on a shaker at 37 °C. During incubation, optical density of the culture was measured every 20 minutes using optical spectrometer. When the optical density of the culture reached between 0.6 – 0.8, 0.5 mM isopropyl β-d-1-thiogalactopyranoside (IPTG) were added to induce transcription. The induction was carried out for 3 hours total on a shaker at 37 °C. During the induction, samples were collected at three time points: at 0 hours, 1 hour, 3 hours. The 0 hours time point was used as control and the 1 hour, 3 hours time points were used to observe the levels of expression induction as time progresses. At each time point 800 µl of the culture were collected and prepared for SDS-PAGE and western blotting analysis. For the final protein isolation, the induction of gene expression was carried out in 1 l of LB medium, therefore, the volume inoculated was 20 ml of the grown culture. After 1 hour of induction, the whole culture was pelleted and stored at -20 °C.

4.13.4 Isolation of protein

The isolation of protein was carried out under denaturing conditions, the composition of all buffers that were used are stated in Table 9. Buffers stored in the fridge were first tempered to room temperature and their pH was adjusted. Affinity chromatography using Ni-NTA agarose (Bio-Rad) for His-tagged proteins was carried out with the ABC1 antigen gene cloned into pET-42b (+). The pelleted culture of the induced BL21 was resuspended in no more than 20 ml buffer B, and thereafter cell lysis was performed using sonication (2x1 minute, 60 % amplitude). The full lysate was centrifuged in the Optima XPN-90 Ultracentrifuge (Coulter Beckman) (100 000 g, 15 minutes, at room temperature). An SDS-PAGE sample was collected from both pellet and supernatant. The supernatant was slowly poured onto the Ni-NTA column and left to drip freely for the protein to properly bind. The flow-through was collected. The column was washed twice with 4 ml of buffer C. The protein was eluted four times with 0.5 ml of buffer D, four times with 0.5 ml of buffer E and four times with 0.5 ml of buffer I. All washes and elutions were collected and 10 µl of each fraction were prepared for SDS-PAGE analysis.

Table 9. List of buffers used for denaturing isolation of recombinant protein.

Buffer	Composition
Buffer B – lysis buffer	100 mM NaH_2PO_4 10 mM Tris-Cl 8 M urea pH 8.0
Buffer C – wash buffer	100 mM NaH_2PO_4 10 mM Tris-Cl 8 M urea pH 6.3
Buffer D – elution buffer	100 mM NaH_2PO_4 10 mM Tris-Cl 8 M urea pH 5.9
Buffer E – elution buffer	100 mM NaH_2PO_4 10 mM Tris-Cl 8 M urea pH 4.5
Buffer I – elution buffer	50 mM NaH_2PO_4 300 mM NaCl 450 mM imidazole pH 8.9

For higher yield of protein, the isolation from inclusion bodies protocol was followed during the isolation of the ABC2 antigen. The pelleted culture was resuspended in 5 ml of buffer (20 mM Tris, 20 mM NaCl, pH 8), and the cells were lysed with sonication (2x 1 minute, 60 % amplitude). The lysate was then centrifuged using a table centrifuge (20 000 g, 20 minutes, 4 °C). The pellet was washed twice (20 mM Tris, 2 % Tween, 2 M urea, pH 8.6), and samples for SDS-PAGE analysis were collected.

4.13.5 Immunisation

The purified protein was loaded into an SDS-PAGE gel and stained with Coomassie Briliant Blue. One visible bend was cut out from the gel and homogenized with Dounce homogenizer. The protein-gel suspension was divided into three 2 ml shots, each containing 0.2 mg of the antigen. A rat was immunised three times, one month apart.

4.13.6 Testing function of antibodies

A blood serum from the immunised rat was collected and stored at -20 °C. Western blots with full *A. castellanii* cell lysates, membrane and cytosolic fractions were prepared and incubated with the blood serum (1:100 dilution) overnight at 4 °C.

The expression of the ABC transporter in *A. castellani* was upregulated to multiply the amount of protein on the western blots. To upregulate the ABC transporter, *A. castellanii* cells were cultivated in 6.25 µM PEITEC for 24 hours. Their harvest was followed by preparing the cell lysate and the membrane fractions.

The membrane and the cytosolic fractions were prepared using sonication (2x1 minute, 60 % amplitude). This lysate was centrifuged (1 500 g, 10 minutes) to pellet all the intact cells. The supernatant was again centrifuged at 20 000 g for 10 minutes to pellet the membrane fraction.

4.13.7 Antibody clean-up

A 1 cm^2 square of nitrocellulose membrane was placed into a glass petri dish and covered with the purified antigen diluted in PBS (1 mg/ml). The membrane with antigen was incubated for 30 minutes at room temperature. Subsequently the membrane was cut into approximately 1 mm^2 pieces using a scalpel and transferred into a plastic microtube. A blocking buffer (PBS, 0.01 % Tween 20, 3 % BSA) was added into the tube and it was incubated on a rotator for 1 hour at room temperature. The tube was centrifuged (14 000 g, 5 minutes) to pellet the membrane and the supernatant was discarded. The antibody containing serum was diluted in PBS (1:4) and 1 ml of the mixture was added into the pelleted membrane, which was incubated overnight at 4 °C.

The membrane was pelleted (14 000 g, 5 minutes) and washed in 1 ml of wash buffer (PBS, 0.01 % Tween 20) for 2 minutes at a rotator a total of two times. Subsequently the membrane was washed in 1 ml of PBS for 5 minutes a total of three times. The antibodies were eluted with 100 µl of elution buffer (100 mM Glycine HCl, pH 2.5). The membrane with elution buffer was pelleted and the supernatant was aspirated and transferred into a new plastic microtube. The elution step was repeated three times. The pH level was adjusted in each of the elution fractions to neutral, using 1M Tris pH 11 and a pH indicator paper.

4.14 Genetic modification of *A. castellanii*

To test the functional properties of the ABC transporter, *A. castellanii* cells were modified by reverse genetics or by gene overexpression. These techniques utilized chemical transfection of *A. castellanii.*

4.14.1 Transfection of *A. castellanii*

All transfections were carried out in a 6-well plate in sterile conditions. The amount of $5 \cdot 10^5$ cells of *A. castellanii* from a culture flask was placed in each well and incubated for 30 minutes at room temperature in order to allow the cells to attach to the bottom of the well.

Simultaneously, a DNA sample was prepared for transfection. When transfecting a plasmid, the plasmids ought to be incubated for 10 minutes at 70 °C and then cooled down at room temperature. 2 µg of DNA were diluted in 100 µl of encystment medium (20 mM Tris–HCl [pH 8.8], 100 mM KCl, 8 mM $MgSO_4$, 0.4 mM $CaCl_2$, 1 mM $NaHCO_3$). A reaction mixture without any DNA was used as control.

Thereafter, 10 µl of PolyFect (Qiagen) were added to the DNA mix, vortexed for 10 seconds and incubated for 10 minutes at room temperature. During the incubation, the medium was aspirated from the cells and substituted by 3 ml of fresh PYG medium. After the DNA mix incubation was finished, 600 µl of PYG medium were added to the DNA mix and the whole mix was immediately added to the wells. By gently swirling the plate, the DNA mix got well distributed within the medium. The transfection plate was incubated over night at 27 °C.

The following day the medium was replaced by a fresh PYG medium with 8 µl/ml of geneticin and left to grow and form a monolayer. After approximately a week the antibiotics concentration was raised to 15 µg/ml, and then more antibiotics were added gradually based on the cell growth, until the final concentration was reached (50 µg/ml).

4.14.2 Overexpression of ABC transporter in *A. castellanii*

Plasmids expressing GFP-tagged proteins (pTPBF, pGAPDH) were utilized to confirm the localisation of the ABC transporter in *A. castellanii.* The plasmid constructs contained either TATA-Binding Protein (TBP): TBP Promoter Binding Factor (TPBF) or Glyceraldehyde Phosphate Dehydrogenase (GAPDH) gene promoters from *A. castellanii* (Bateman, 2010). The cDNA sequence of the ABC transporter was inserted into the plasmid using restriction sites (BglII and Ndel). All plasmids were sequenced and subsequently transfected to *A. castellanii* cells. An empty plasmid was also transfected as control. After establishing a growing culture, the transfected cells were prepared for microscopy analysis.

4.14.2.1 Fluorescent microscopy

The transfected *A. castellanii* cells were left to attach for 30 minutes at room temperature using a 6-well glass bottom plate (Cellvis). The Leica TCS SP8 WLL SMD-FLIM microscope (Leica, Germany) equipped with an HC PL APO CS2 63x/1.20 water objective was used for observation of fluorescence in the living cells. The excitation the GFP was set at 488 nm and the emission at 498-551 nm.

4.14.3 Knockout via cassette integration

The knockout of the ABC transporter was carried out by inserting a selection marker into the genome and therefore disrupting the targeted gene. The selection marker was integrated into the genome via homologous recombination within the untranslated regions (UTR) regions of the ABC transporter gene.

4.14.3.1 Cassette design

The cassette and all the primers for amplification were designed using the Geneious Prime software. The cassette consisted of three parts: 5´UTR, an antibiotic resistance and 3´UTR (Figure 8). Geneticin and hygromycin resistance were used as selection marker and their sequences contained all the means for protein production. The UTR regions of the ABC transporter were acquired using AmoebaDB, with each sequence being 1kb long.

Figure 8. ABC transporter knockout cassette. Abbreviations: UTR – untranslated regions, SV40 – *Simian* virus 40 promotor, TBP – TATA binding protein promotor, Gen – geneticin resistance gene, Hyg – hygromycin resistance gene, PolyA – polyadenylation signal.

All the parts were acquired using PCR (Q5 High Fidelity 2X Master Mix). The primers for the amplification of each of the three parts contained overhangs to the neighbouring part (Table 10). The reverse primers are the reverse complements to the forward primers of the neighbouring part to their right. This helps a better annealing of the three parts during the assembly of the cassette (Figure 9).

Table 10. Primer sets for knockout cassette. In bold: overhangs to the neighbouring region.

	Region	Forward primer	Reverse primer
Geneticin resistance	5´ UTR	5´ GCCGACATCTCTTGCAATGA	5´ **CTGGGGACTTTCCACACC**GAGTTCTTGGAAGAGCGTGT
	Resistance gene	5´ **ACACGCTCTTCCAAGAACTC**GGTGTGGAAAGTCCCCAG	5´ **TGAACAACGTGGTATGTGGCT**CACACAAAAAACCAACACACAGAT
	3´ UTR	5´ **ATCTGTGTGTTGGTTTTTGTGTG**AGCCACATACCACGTTGTTCA	5´ CCAACTCATCAGACGATTCAG
Hygromycin resistance	5´ UTR	5´ CGGTGGCCGACATCTCTT	5´**CTGGGGACTTTCCACAC**CCGAGCAGGAGCGTCATCT
	Resistance gene	5´ **AGATGACGCTCCTGCTCG**GGTGTGGAAAGTCCCCAG	5´ **GAACCGAGATAATGAGCCC**CACACAAAAAACCAACACACAGATGTAAT
	3´ UTR	5´ **ATTACATCTGTGTGTTGGTTTTTTGTGTG**GGGCTCATTATCTCGGTTC	5´ ATTACATCTGTGTGTTGGTTTTTTGTGTGGGGCTCATTATCTCGGTTC

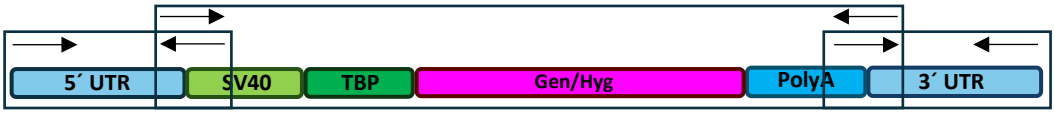

Figure 9. ABC transporter knockout cassette with assembly primers (arrows). Dark blue rectangles show parts of cassette synthesised before complete assembly. Abbreviations: UTR – untranslated regions, SV40 – *Simian* virus 40 promotor, TBP – TATA binding protein promotor, Gen – geneticin resistance gene.

4.14.3.2 Cassette assembly

The amplicons of the UTR regions and the resistance cassette were assembled with the use of PCR with a forward 5´UTR primer and a reverse 3´ UTR primer utilising the Q5 High Fidelity 2X Master Mix (New England Biolabs). The final purified product was ligated to pJET 1.2/blunt. Following the standard protocol, the ligation reaction was transformed into TOP10 *E. coli* strain, and the plasmids were isolated.

Before the transfection to *A. castellanii*, the plasmid was linearized using the BglI restriction enzyme. *A. castellanii* cells were first transfected with a cassette containing geneticin resistance and after establishing the knockout cell line and confirming cassette integration, the second transfection was performed with hygromycin resistance cassette.

4.14.3.3 Genotyping of knockout *A. castellanii* strains

The established knockout cell lines were sorted into a 96-well plate on BD FACS Aria Fusion (BD BioSciences), using the single cell sorting technique. The surviving clones were cultivated in 27 °C. The genomic DNA was isolated from the clones using the E.Z.N.A. Tissue DNA Kit (Omega Bio-Tek). All genotyping was executed using PCR with specific primers to cover regions of integration, as shown on Figure 10.

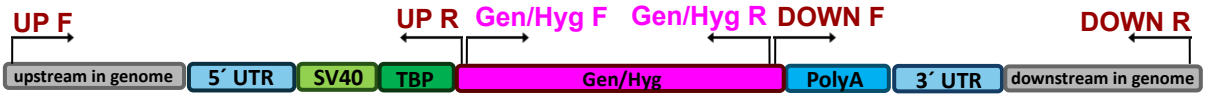

Figure 10. Cassette prepared to create knockout stains of *A. castellanii,* and primers used to test its correct integration. Abbreviations: UTR – untranslated regions, SV40 – *Simian* virus 40 promotor, TBP – TATA binding protein promotor, Gen – geneticin resistance gene, Hyg – hygromycin resistance gene, PolyA – polyadenylation signal. Primers: UP F/R - forward and reverse primers for amplification of upstream genomic region to the geneticin/hygromycin resistance, Gen F/R - forward and reverse primers for amplification of the geneticin/hygromycin resistance, DOWN forward/reverse - forward and reverse primers for amplification of geneticin/hygromycin resistance to the downstream genomic region.

4.14.4 Knockout via CRISPR Cas9

The CRISPR Cas9 protocol was carried out in accordance with Philippe et al., 2024.

4.14.4.1 Cas9 plasmids

Plasmids for CRISPR Cas9 were provided by Hugo Bisio (French National Centre for Scientific Research). A single plasmid was sufficient for expressing both Cas9 endonuclease and polycistronic guide RNA in *A. castellanii*. The DNA sequences for the gRNA expression were

cloned in a polycistronic cassette to increase knockout effectiveness. Scaffolds for the gRNA expression were designed to contain *A. castellanii* specific U6 promotor and a tRNA-gRNA-tracerRNA sequence under the promoter. The vHB16 plasmid (Figure 11) contained two tRNA-gRNA-tracerRNA sequences under one U6 promotor, which was also used as template for new gRNA cassettes. The vHB8 plasmid (Figure 12) was generated by replacing gRNA1-tracerRNA1-gRNA2 by a NotI restriction site (Bisio et al., 2023), in which new gRNA cassetes were inserted.

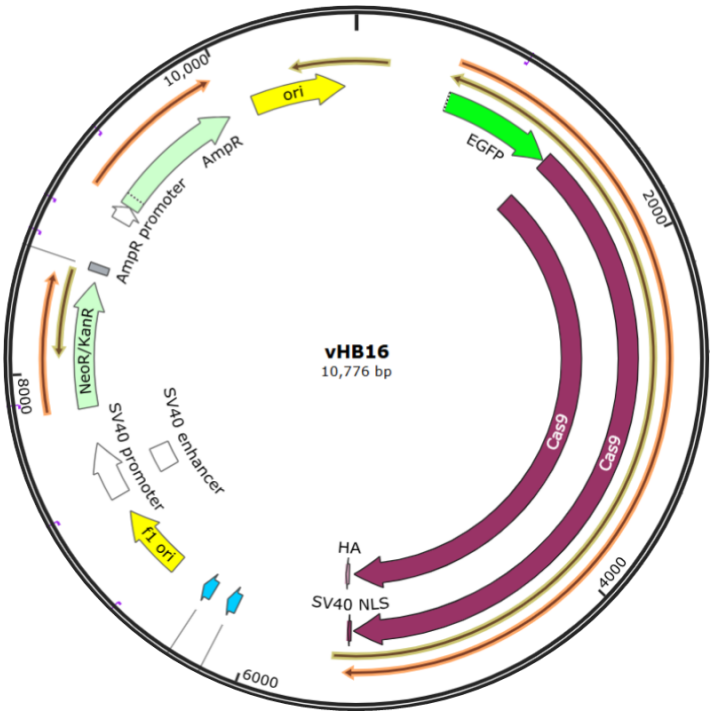

Figure 11. Plasmid vHB16 encodes Cas9 and gRNA scaffolds for polycistronic expression in *A. castellanii*.

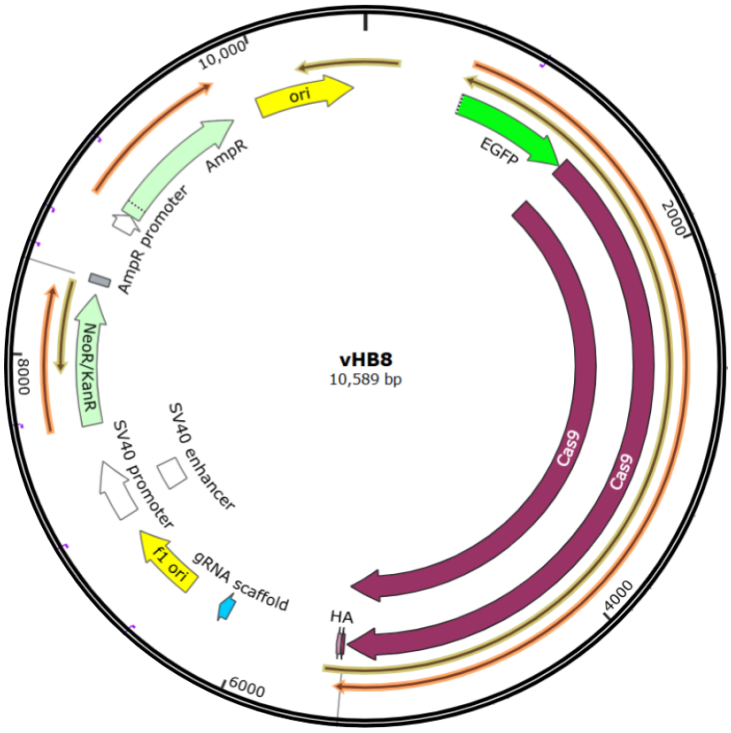

Figure 12. Vector vHB8 for Cas9 and gRNA expression in *A. castellanii*.

4.14.4.2 Guide RNA design

To verify the functionality of the Cas9 system, a cellulose synthase (ACA1_349650) knockout was carried out as in Bisio et al., 2023. Sufficient gRNAs for ACA1_349650 were selected from the VEuPathDB CRISPR guide design tool (http://grna.ctegd.uga.edu/). All the used gRNA are listed in Table 11.

Table 11. Guide RNAs designed for each gene or gene group.

Gene number	gRNA sequence	
ACA1_349650	gRNA1	GAGAACGCGTGCGCCTTCGC
	gRNA2	GCCGTCAAGATCCTGGCCAC

4.14.4.3 Cloning of gRNAs

Mutagenesis of the Cas9 plasmids was selected as a cloning strategy. The primer sequence included gRNA and homology sequences to vHB16, which was used as a template (Table 12). The 2x Phusion Green Hot Start II High Fidelity PCR Master Mix (Thermo Scientific) was used for the PCR reaction with 20 ng of template (vHB16) in a total volume of 20 µl. After the amplification the product was separated on agarose gel and then isolated from the gel.

Table 12. Primers used for gRNA cloning, containing homology arms to vHB8 plasmid and N sequence represents gRNA.

Primer	Specification
TCCCCATACTGGCCANNNNNNNNNNNNNNNNNNN GTTTTAGAGCTAGAAATAGC	gRNA cloning primer for vHB16, forward
TTCTAGCTCTAAAACNNNNNNNNNNNNNNNNNNNT GGCCAGTATGGGG	gRNA cloning primer for vHB16, reverse
GTTGTTCCGGGTCGACTCTAG	Colony screening primer for vHB8

To finish cloning, the vHB8 plasmid was cleaved with NotI, dephosphorylated by Shrimp Alkaline Phosphatase (SAP) (Thermo Scientific) according to manufacturer protocol, and heat inactivated at 65 °C for 15 minutes.

The purified PCR product with gRNAs was ligated to the cleaved vHB8 plasmid via Gibson cloning (chapter 4.4). It was subsequently transformed into TOP10 *E. coli* strain and plated on an agar plate with ampicillin. A number of colonies from the agar plate was selected and the protocol for plasmid isolation was followed (chapter 4.9). The purified plasmids were sequenced and evaluated before the transfection into *A. castellanii*.

4.14.4.4 Cas9 localisation microscopy

The live cells were observed with the Nikon CSU-W1, Yokogawa CSU-W1 spinning disk module (Nikon Instruments Inc.) equipped with a CF Plan Apo VC 60XC WI 60x magnitude water objective. The cells were observed in dH_2O in 35 mm glass bottom petri dishes.

4.14.4.5 Hoechst staining

The live cells were incubated in 1:2000 Hoechst solution (Thermo Scientific) for 1 hour at room temperature, washed tree times in dH_2O and placed in a 6-well plate to attach.

4.14.4.6 Genotyping of CRISPR Cas9 *A. castellanii* strains

The genomic DNA from the knockout strains was isolated using the E.Z.N.A. tissue DNA kit (Omega Bio-tek). Primers for the genotyping were designed to cover the target area of the genome using the Geneious Prime software. The primers were designed to amplify sequences no longer than 1 kb. The PCR was preformed using the 2x Phusion Green Hot Start II High Fidelity PCR Master Mix (Thermo Scientific) and purified with magnetic carboxylated beads (BOMB.bio). The PCR product was ligated to pJET via blunt ends and transformed into TOP10 *E. coli* strain. Ten colonies were picked for further analysis. The plasmid was extracted from the bacteria using the E.Z.N.A. Plasmid DNA Mini Kit I (Omega bio-tek). The isolated plasmid was sequenced and analysed in Geneious Prime in order to identify any deletion or changes in the sequence.

4.14.4.7 Phenotyping of the cellulose synthase knockout

The cellulose synthase knockout exhibited defect in cyst formation, therefore, the phenotype was observed in cells, which have undergone encystation. The knockout strain was incubated in encystment medium (20 mM Tris–HCl [pH 8.8], 100 mM KCl, 8 mM $MgSO_4$, 0.4 mM $CaCl_2$, 1 mM $NaHCO_3$) for 5 days or until no trophozoites are present. The wild type strain was used as a control for the completion of encystation.

After the encystation was completed, the one drop Calcofluor white stain (Sigma-Aldrich) was added directly to the encystment medium, and the sample was immediately proceeded to microscopic examination using the Leica TCS SP8 WLL SMD-FLIM microscope (Leica, Germany) equipped with an HC PL APO CS2 63x/1.20 water objective. The samples were examined with a UV light laser set at excitation 358 nm and emission 461 nm.

4.14.5 Half maximal inhibitory concentration assay

Any phenotype changes of ABC transporter knock out strains were studied in a half maximal inhibitory concentration (IC_{50}) assay. The half inhibitory concentration was measured in a 96-well plate using two selected drugs – amphotericin B, phenethyl isothiocyanate (PEITC).

Stock solutions of the drugs were prepared as followed. Amphotericin B was diluted in DMSO creating a 50 mM stock solution. PEITC was diluted with ethanol to prepare 5 mM stock solution.

A series of two-fold dilutions (in 12 wells) was set up in 100 µl of PYG media. The starting concentration of amphotericin B was 250 µM and the final concentration of DMSO was no more than 2 %. As control, the same procedure was repeated with only DMSO (without any diluted Amphotericin B). As for PEITC the experiment was set up with starting concentration at 200 µM.

The volume of 100 µl of 20000 cell/ml in PYG media was added into each well, to reach the final volume of 200 µl. The cell density was measured using Z2 Coulter (Beckman Coulter) with measuring thresholds set at the upper size Tu 7 µm and the lower size Tl 21 µm and dilution factor 100.

All plates were incubated for 72 hours at 27 °C.

4.14.5.1 Experiment analysis and statistical analysis

The *A. castellanii* cells were placed on ice for 30 minutes to detach from the bottom of the wells. They were then fixed with 4 % formaldehyde for 20 minutes at room temperature. The cell concentration in each well was measured by the Guava EasyCyte 11HT flow cytometer (Luminex).

Each experiment was repeated at least three times to create a suitable data set for statistical analysis. The data obtained from the flow cytometer was processed in GraphPad Prism and the half inhibitory concentration was calculated using nonlinear regression. Comparisons of knockout and wild type strains were performed with one-way ANOVA test and Tukey's multiple comparisons test in GraphPad Prism.

4.15 Quantitative PCR

4.15.1 Drug response analysis

A culture of *A. castellanii* cells was set up by inoculating 30000 cells per millilitre in 5 ml of PYG medium. After two days of growth at 27 °C, the medium was removed from the flasks and replaced with a fresh medium supplemented with drugs (Table 13). Furthermore, each flask and each control contained 0,5 % of ethanol. All conditions were prepared in tetraplicates.

Table 13. Concentrations of drugs used for qPCR analysis.

Drug	Concentration [µM]
Amphotericin B	8
Rotenone	6.25
PEITC	50

The cells were harvested after 2 and after 8 hours of incubation at 27 °C and washed three times in PBS. Altogether, with 3 conditions in tetraplicates and two time points, 24 culture flasks were prepared. The pelleted cells were then stored at -80 °C.

4.15.2 qPCR sample preparation

Cultures set up in tetraplicates were harvested and washed three times with PBS. The pelleted cells were then stored at -80 °C.

An *A. castellanii* culture was washed three times with PBS and resuspended in 200 µl of PBS. Total RNA was isolated using the High Pure RNA Isolation Kit (Roche) and stored at -80 °C.

The reactions were set up into a 96-well plate using the SYBR Green One Step qRT-PCR Kit (KAPA). Each reaction contained 100 ng of RNA in a volume of 10 µl. The ß-actin (ACA1_151070) housekeeping gene was used as endogenous gene control (Grechnikova et al., 2022), and all primers for this experiment are stated in Table 14.

Table 14. Primers used for qPCR.

Primer	Sequence
ABC transported forward	5´-ACTACTATCAGCGCGACACA
ABC transporter reverse	5'-AGTTGAGGCCAGTCATCCAA
ß-actin forward	5'-CTGCAGCAAGTGCTACTGAG
ß-actin reverse	5'-AAATACCATTCGCGCAACCA

The qPCR programme (Table 15) was set up in CFX96 Real time PCR instrument (Bio-Rad).

Table 15. Program settings for qPCR.

Cycle	Temperature	Time
Reverse transcription	42 °C	30 minutes
Initiation	95 °C	5 minutes
Cycle 39x	95 °C	10 seconds
	56 °C	20 seconds
	72 °C	20 seconds
Termination	55 °C	5 seconds
	95 °C	1 minute

Data analysis was performed in Excel to normalize the data and to calculate fold change using formula $2^{(-\Delta\Delta C_T)}$. Statistical analysis was performed in GraphPad Prism using ANOVA statistical test.

5 Results

5.1 Response to stress conditions

Proteomic analysis of *A. castellanii* regarding the response to oxidative stress conducted by Ženíšková et al. (2025) demonstrated significant upregulation of the ABC transporter in oxidative stress conditions caused by PEITC and Rotenone. PEITC cause malfunction of mitochondria or increase ATP production, and it is mostly used as an anticancer drug (Keum et al., 2003; Zhen et al., 2023). Rotenone is an inhibitor of complex I in the electron transport chain, which particularly inhibits transport of electrons to ubiquinone (Heinz et al., 2017). To confirm the proteomic results, a qPCR analysis was performed with 3 conditions, in which the increase of ABC transporter was observed. In addition, the drug amphotericin B was used to compare different mechanism other than generation of oxidative stress. Amphotericin B is an antimycotic, also used as a treatment for leishmaniasis. Its major effect is disintegration of the cytoplasmic membrane (Anuntasomboon et al., 2024; Janik et al., 2023).

Samples were collected after 2 and 8 hours after treatment and their changes in the ABC transporter levels were observed (Table 16, Figure 13). However, only in PEITEC, they were statistically significant (Figure 13). Transcription levels elevated in both time points PEITC condition. This corresponds with the proteomic analysis, where in PEITEC the protein level increased 8-fold and 19-fold at 2 and 8 hours, respectively, whereas the number of transcripts increased 18-fold at 2 hours and 71-fold at 8 hours. In rotenone the protein level increased 1.6-fold and 1.77-fold at 2 and 8 hours, respectively. The qPCR analysis of rotenone showed almost no change in transcription at 2 hours, however, at 8 hours increased by 1.47 (Table 16), however, this change in transcription level was not statistically significant.

As for amphotericin B an increase was observed at 2 hours, where the transcription almost doubled and at 8 hours the transcription remained elevated, although it decreased compared to 2 hours incubation (Table 16).

Table 16. qPCR analysis of the response to amphotericin B, rotenone, PEITEC, which was prepared in 2 and 8 hours of incubation. Data are presented in fold change against the wildtype, ± standard deviation.

fold change		
	2 hours	8 hours
Amphotericin B	1.83 ± 0.23	1.37 ± 0.7
Rotenone	1.03 ± 0.10	1.47 ± 0.05
PEITEC	18.21 ± 1.03	71.3 ± 4.85

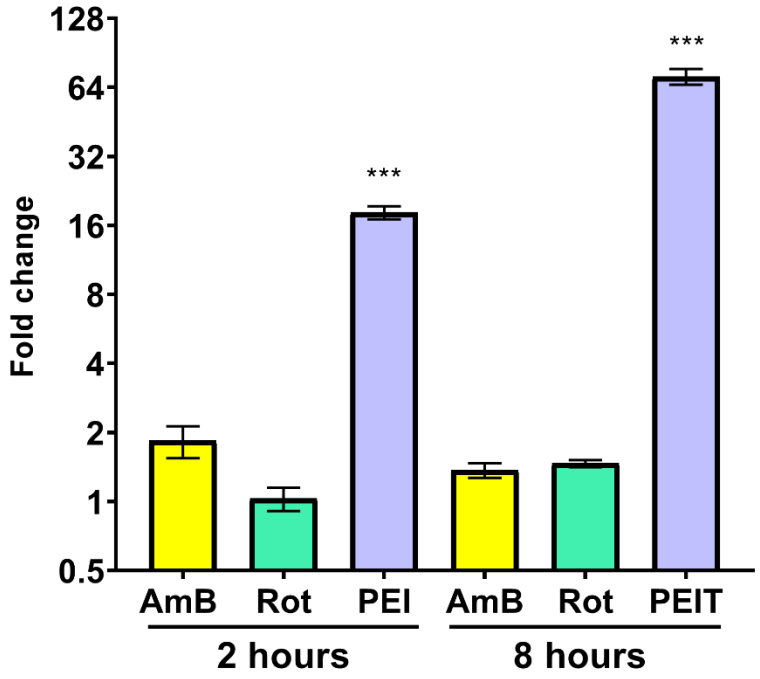

Figure 13. Graphical representation of ABC transporter fold change in the qPCR analysis of the response to amphotericin B, rotenone, PEITEC. The fold change values (y-axis) are in log_2 scale. *** - p-value <0.001

5.2 Localisation of ABC transporter

To characterise the ABC transporter, we conducted an experiment to localised it in a specific cellular compartment. Confirmation of the localisation of proteins without specific antibodies can be executed using a GFP-tagged protein and observing its fluorescence in cellular compartments. The ABC transporter was predicted to be localised in the cytoplasmic membrane, due to its predicted structure and presumed function as a cellular exporter (Figure 14). Based on the topology prediction, the ABC transporter has 12 transmembrane helices forming two transmembrane domains, each containing six helices. These domains are separated by a cytosolic domain and on the N-terminus the ABC transporter carries another cytosolic membrane, which results in a half symmetrical quaternary structure. This generally corresponds to the structure of ABC transporters, that usually possess two cytosolic membranes/NBDs and two transmembrane domains/TMDs.

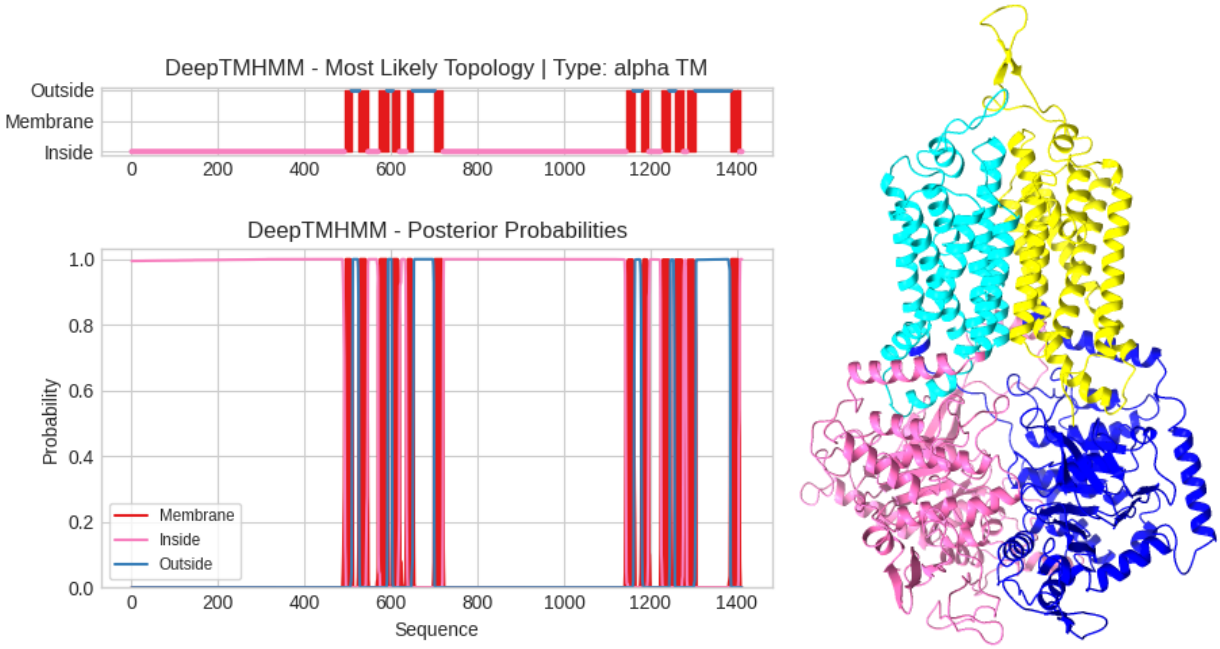

Figure 14. Structural prediction and topology of domains of the ABC transporter provided by DeepTMHMM on the left. The diagram shows the probability of amino acid sequences to be present in the cytosol (pink), membrane (red) or outside of the cell (blue). On the right is the structural model of the ABC transporter executed by AlphaFold3. TMDs are highlighted in yellow and cyan each colour representing one half of the protein. The NBDs are highlighted in pink and blue representing the two NBDs interacting upon ATP binding.

On Figure 15 is the localisation of the GFP-tagged ABC transporter, which was expressed using two promotors of different strength. The localisation is clear on the cytoplasmic membrane, when compared to the control. The GAPDH showed a strong fluorescent signal in the cytoplasm, although this can be caused by the high expression of the GAPDH promotor and the inefficiency of transport to its final compartment (middle panel on Figure 15).

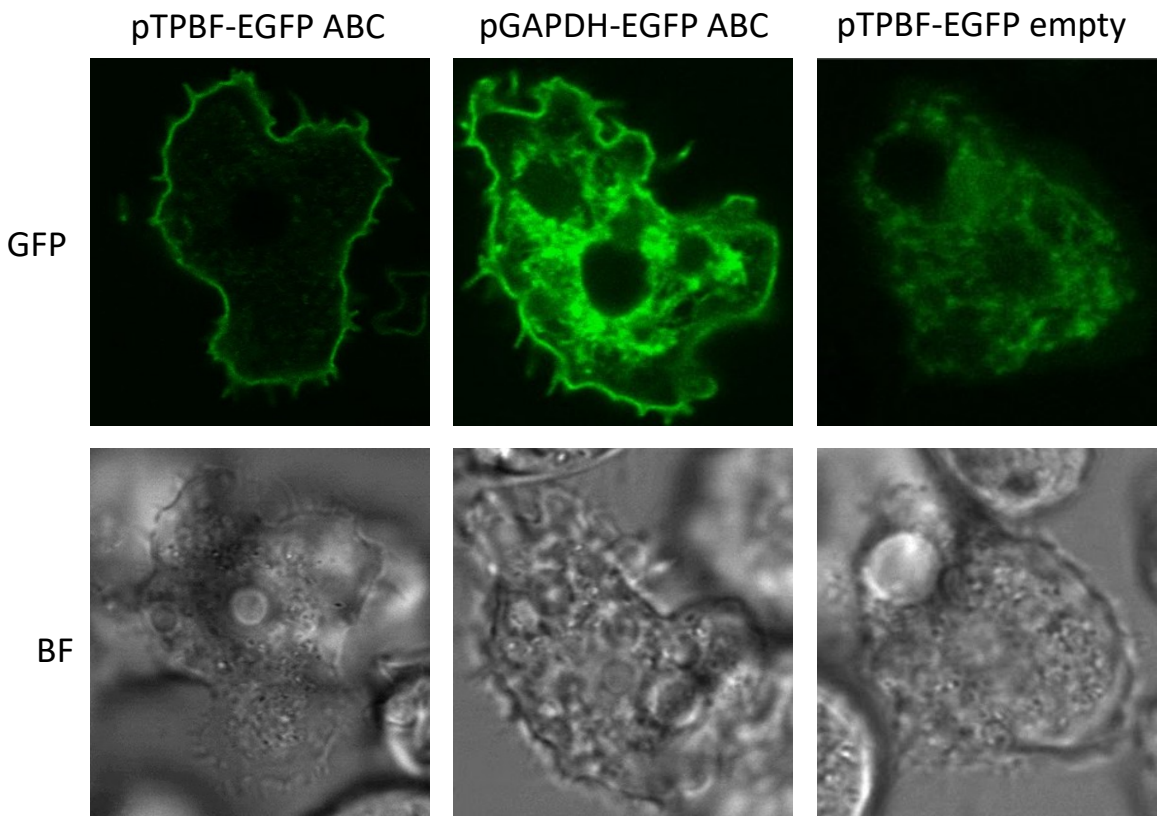

Figure 15. Localisation of the ABC transporter with GFP-tag. ABC transporter is expressed under different promotors: TPBF – moderate level of expression, GAPDH – high level of expression. Plasmid containing only GFP gene under TPBF promoter was used as control. Abbreviations: BF – bright field

As an additional experiment, transcription level of the GAPDH over expressing strain was tested. The transfected cultures usually show a low number of fluorescent cells and therefore the transcription was tested by qPCR for following experiments. The 120-fold elevation of transcription confirmed its suitability for further experiments (Figure 16).

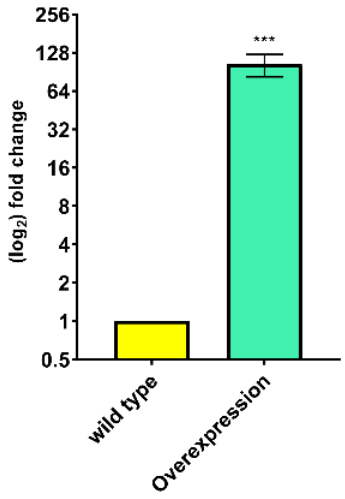

Figure 16. qPCR analysis of the transfected cells with plasmid for ABC transporter overexpression. *** p-value <0.001

5.3 Knockout via cassette insertion

Reverse genetics is an important tool to assess the function of various genes. Herein, the substitution of the gene of interest for a selection marker was chosen to produce a knockout of the ABC transporter.

5.3.1 Knockout confirmation

After a successful synthesis of the knockout cassette and selection of the transfected *A. castellanii* cells, it was necessary to confirm the correct integration of the cassette into the genome. This was carried out with PCR using multiple primers to amplify unique sequences to the integrated cassette into the ABC transporter gene, as visualized on Figure 17, panel A.

The geneticin resistance gene was introduced into *A. castellanii* cells as the first selection marker and tested separately before introduction of other selection markers. The geneticin cassette was integrated well into the genome with very strong signal in the resistance gene itself. This could be caused by the residual resistance cassette, which presence on episomes after transfection. Even though the signal on the agarose gel of the cassette integration was moderate to weak, it is a proof of integration into the ABC transporter gene, because the amplified sequences are unique to the knockout strain, as shown on Figure 17, panel B. Similar situation was observed after the transfection of the second cassette, which is the hygromycin resistance gene. In both cases, a control gene was used to test the quality of the *A. castellanii* genomic DNA and PCR reaction itself (Figure 17).

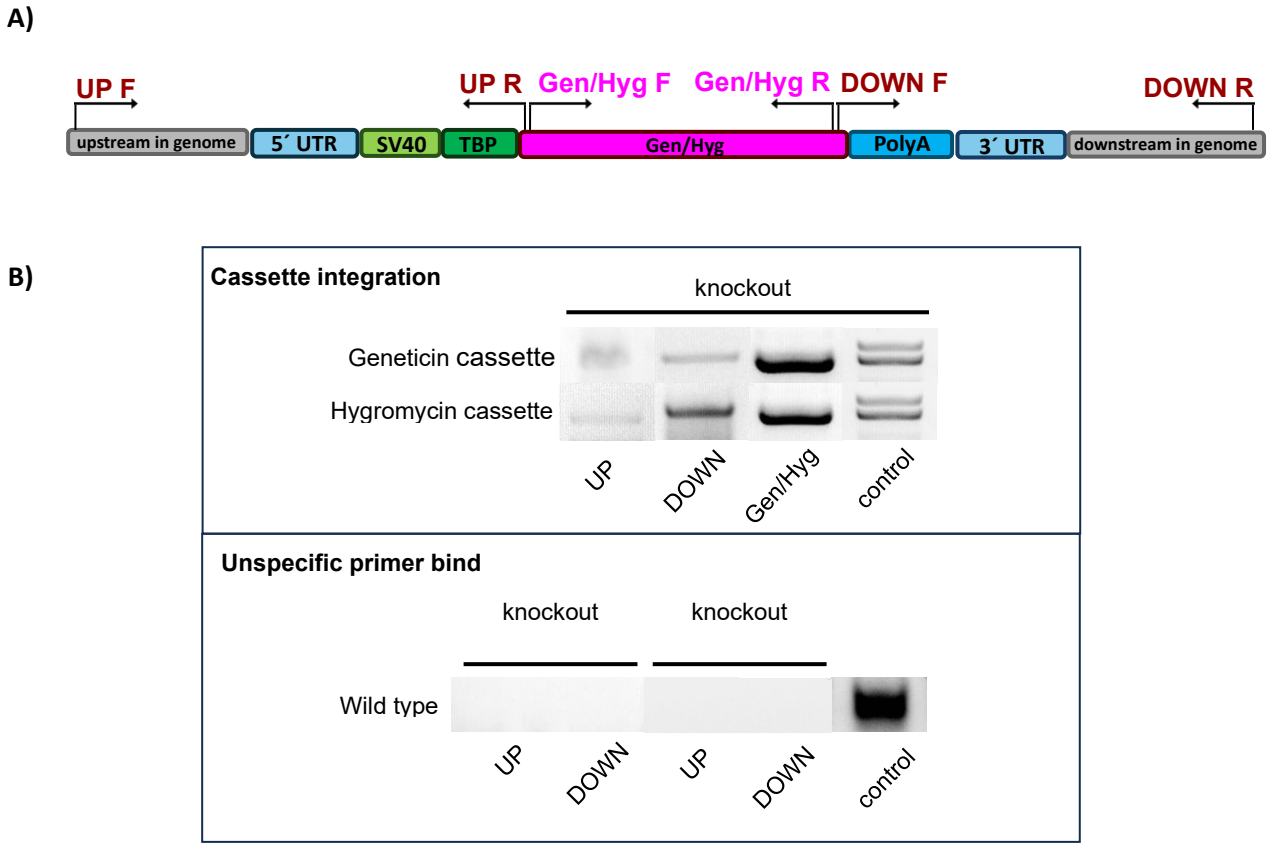

Figure 17. Inspecting proper cassette integration in genome. **A)** Cassette prepared to create knockout strains of *A. castellanii,* and primers used to test its correct integration. Abbreviation: UTR – untranslated regions, SV40 – *Simian* virus 40 promotor, TBP – TATA binding protein promotor, Gen – geneticin resistance gene, Hyg – hygromycin resistance gene, PolyA – polyadenylation signal, primers: UP F/R - forward and reverse primers for amplification of upstream geneticin/hygromycin resistance genomic region, Gen F/R - forward and reverse primers for amplification of the geneticin/hygromycin resistance, DOWN forward/reverse - forward and reverse primers for amplification of the downstream geneticin/hygromycin resistance genomic region. **B)** Cassette integration: DNA gel showing the integration of knockout cassettes into *A. castellanii* genome. PCR products are corresponding with primer regions in panel A. An *A. castellanii* gene, which is known to be expressed, was used as control. Unspecific primer bind: Confirmation of specific primer binding. Wild type strain has been used to test cassette specific primers paired with genome specific primers, as was used in panel B for both geneticin and hygromycin cassette. PCR products are corresponding with primer regions in panel A.

A mere confirmation of integration into the genome is not sufficient for a knockout strain. The next step was to investigate the presence of undisrupted ABC transporter gene, which was carried out with PCR and primers, which are annealing in the wild type gene (Figure 18, panel A). Testing with different sets of primers showed, that the ABC transporter gene is still present in the genome, despite the correct integration of selection marker cassette (Figure 18, panel B). *A. castellanii* genome is polyploid, which might cause difficulties to fully knockout a gene (Byers, 1986) and only leads to a partial knockout of the ABC transporter gene was generated by this method.

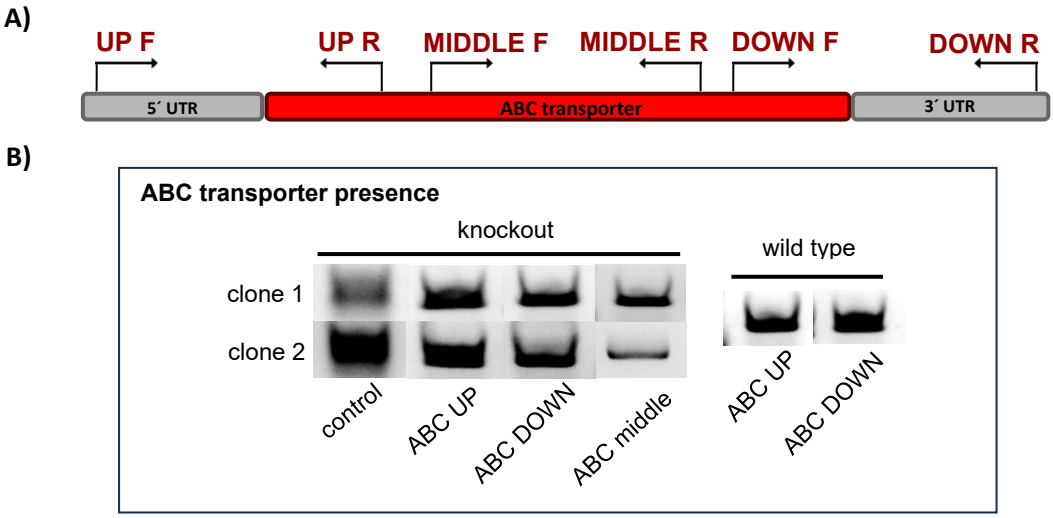

Figure 18. Test for presence of wild type gene in knockout strains. **A)** Schematic diagram of translated region of ABC transporter and untranslated regions (UTR) in genome and primers used to test its presence in knockout stains. **B)** Presence of ABC transporter undisrupted by knockout cassette integration. PCR products are corresponding to primer regions in panel A. Knockout strain and wild type strain was used for testing. An *A. castellanii* gene, which is known to be expressed, was used as control.

5.3.2 Transcript levels

Depletion of alleles could have an influence on the transcription level of the gene, which can be tested by a quantitative PCR analysis. Knockout strains, with only one cassette (sKO) and both cassettes (dKO), were compared with wild type strain of *A. castellanii* by qPCR analysis and fold change was calculated. Fold change shows the ratio between the quantity of the wild type strain and the knockout strain, this means that no change in transcript levels equals 1 and complete absence equals 0.

Changes in transcript levels were observed in both strains, however, the changes were not statistically significant in both cases. This shows that this knockout method has some effect on the gene transcription, but not strong enough to be proven statistically. Strain sKO showed a decrease in transcription of the ABC transporter by half, which corresponds with depletion of alleles. Whereas the strain dKO unexpectedly increased 1.5 times in transcript levels (Figure 19).

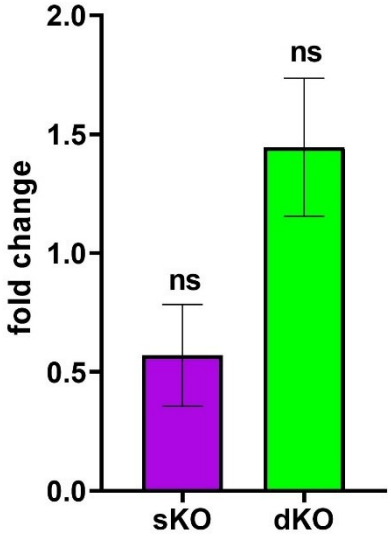

Figure 19. Transcription fold change of the ABC transporter in knockout strains. Abbreviation: sKO – strain transfected with only one selection marker gene, dKO – strain transfected with two selection marker genes, ns - not significant.

5.3.3 Half inhibitory concentration assay

Half inhibitory concentration (IC_{50}) assay was selected to phenotype the knockout strains and to test the response of the ABC transporter to different drugs. IC_{50} provides a concentration, in which the drug achieves its biological effect.

PEITEC and Amphotericin B were tested as a model of different mechanisms of action and with consideration of data shown in chapter 5.1 and with Ženíšková et al., 2025. In this assay strains of *A. castellanii* with different transcription levels of the ABC transporter were used to demonstrate its significance for viability of cells. IC_{50} values were measured on wild type strain, sKO strain, ABC transporter overexpressing strain (OE) and strain containing a plasmid with only geneticin resistance as control (RES) (Table 17). The double knockout strain was excluded due to ambiguous changes in transcription levels.

PEITEC was selected, on the bases of significant effect on wild type *A. castellanii* cells by qPCR and proteomic analysis (Ženíšková et al., 2025). Wild type strain and the RES strain showed significant difference in IC_{50}, which was two times higher in wild type (Table 17). This implies, that cultivation of *A. castellanii* cells with geneticin affects their viability and needs to be considered, when analysing the data. The IC_{50} value in sKO strain is three times lower than in wild type, therefore, even the partial depletion of the ABC transporter shows a significant change in viability of cells enduring PEITEC. However, this result, when compared to the RES strain, shows only a half decrease and is not statistically significant (p-value 0.21). Similar effect was observed in OE strain, where the IC_{50} value was contradictory lower than wild type, although it was more viable than the control strain (Table 17, Figure 20).

Amphotericin B was used for this assay as a control and to use a drug, that does not directly cause oxidative stress. In contrast Amphotericin B did not show any IC_{50} changes, that indicate the involvement of the ABC transporter. The value of IC_{50} raised significantly in the RES strain and a decrease of IC_{50} was observed in sKO and OE strain (Table 17, Figure 20). Consequently, it is likely that Amphotericin B utilizes a different mechanism within the cell and achieves a cytostatic effect rather than cytotoxic effect.

Table 17. PEITEC and Amphotericin B values of IC_{50} measured on different strains of *A.castellanii*. Values correspond with graphs in Figure 20.

	IC50 [µM]			
	PEITEC	± SD	Amphotericin B	± SD
wild type	12.7	± 2.15	8.8	± 1.55
single knockout	3.9	± 1.04	5.6	± 1.05
overexpression	9.4	± 1.04	5.1	± 0.36
ATB resistance	6.5	± 0.90	13.3	± 1.70

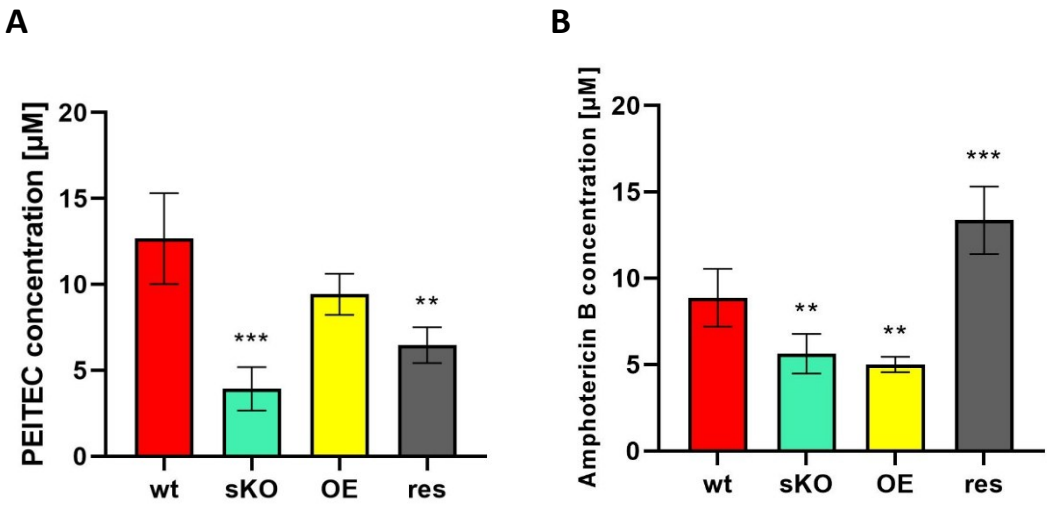

Figure 20. Graphical presentation of IC_{50} values stated in Table 17. Horizontal lines represent the statistical significance of the IC_{50} difference when compared to the wild type strain. Abbreviations: wt – wild type, sKO – single knockout, OE – overexpressing strain, res – strain with plasmid contain only geneticin resistance, ** - p-value < 0.01, *** - p-value < 0.001.

5.4 CRISPR Cas9

As of the date of this thesis's release, only one publication introducing the CRISPR-Cas9 method in *A. castellanii* had been published, by Hugo Bisio and his team (Bisio et al., 2023). Knockout of cellulose synthase gene (ACA1_349650) was replicated in accordance with their protocol to establish this method (Philippe et al., 2024).

5.4.1 Cas9 localisation

Before considering testing for genotype changes and phenotype changes, it is necessary to confirm the localisation of the Cas9 nuclease, which is expected to be present in the nucleus. The

Cas9 nuclease is in this case GFP-tagged, therefore, the fluorescence is a clear indicator of its presence and localisation. On Figure 21 is the GFP signal colocalised with Hoechst stain, which stains DNA.

Another aspect to consider before starting cloning and genotyping is to check the expression of the GFP-tagged Cas9 under the microscope, because the numbers of fluorescent cells vary even in transfections done in parallel with the same plasmid.

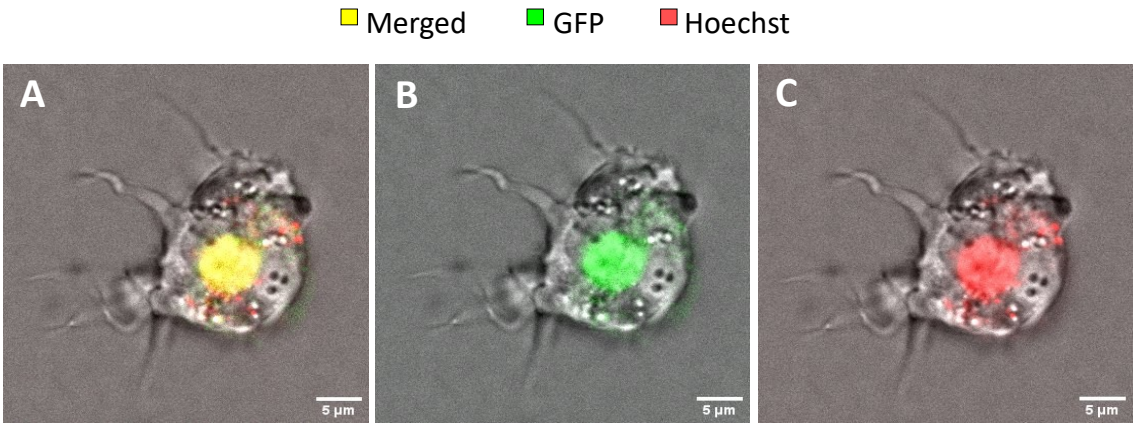

Figure 21. Compartmental localisation of Cas9 in *A. castellanii*. Cas9 tagged with GFP colocalised with Hoechst stain for nucleus.

5.4.2 Genotyping

Determining the genotype and nature of the mutation is essential in the knockout confirmation process. Herein, the genomic sequences complementary to the designed gRNAs were sequenced and searched for any changes. The Cas9 nuclease causes cuts in the genome, which should result in deletions or insertions at the affected site.

The mutations in the cellulose synthase gene are stated in Figure 22. Two gRNAs were used for the cellulose synthase knockout. Two types of mutation were recognized in the gRNA1 cleavage site. A deletion was present in five nucleotides at the 5′ end of the gRNA. An insertion of 17 nucleotides occurred by adding the sequence of the gRNA as a repair after cleavage. The gRNA2 was cleaved at the beginning of the gRNA and four bases were deleted from the genome. This result implies that the process of CRISPR Cas9 was successful, and that the cellulose synthase gene was permanently changed in sequence and the reading frame was shifted.

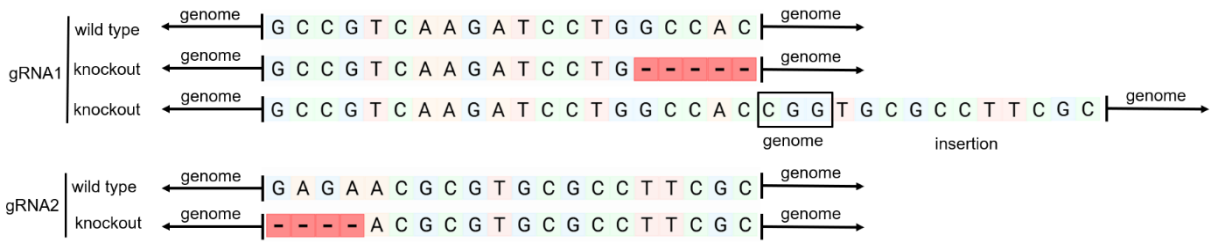

Figure 22. The sequencing results of genotyping of the cellulose synthase knockout. Two gRNAs were designed for this knockout (gRNA1, gRNA2). The results of knockout (KO) sequencing were compared to the wildtype sequence. Highlighted in red are the mutation caused by Cas9 nuclease. Created in BioRender.

5.4.3 Phenotyping

Cellulose synthase has an important role in the synthesis of the cyst wall in *A. castellanii*, since the inner endocyst is composed of cellulose (Moon et al., 2014). Therefore, the phenotype of a knockout manifest during encystment. The cells underwent encystation and were stained with calcofluor, which is a dye used for visualization of cell wall components. When the cyst wall is properly synthesised, a clear signal is visible on the border of the cyst, representing the cyst wall.

A strain with off-target gRNA and expression of Cas9 nuclease was used as control. In addition, a strain with on-target gRNA but without Cas9, was also prepared as control. In Figure 23, panel A the knockout strain cells are rounded and do not resemble a trophozoite, but no clear signal was observed in the cyst wall and only intracellular vacuoles were stained. No cells with a proper cell wall were observed in the knockout strain, which confirms the presence of the functional knockout of the cellulose synthase. Pseudocysts exhibiting the cyst wall staining were observed also in the control strain but their staining differs from pseudocysts present in the knockout strain. This is because cysts do not need to be fully formed for the staining, since the cyst wall is being synthesised at the beginning of the encystation process.

There is a clear difference in shape and localisation of staining between the cysts formed in control and knockout strains, therefore, the knockout phenotype was confirmed.

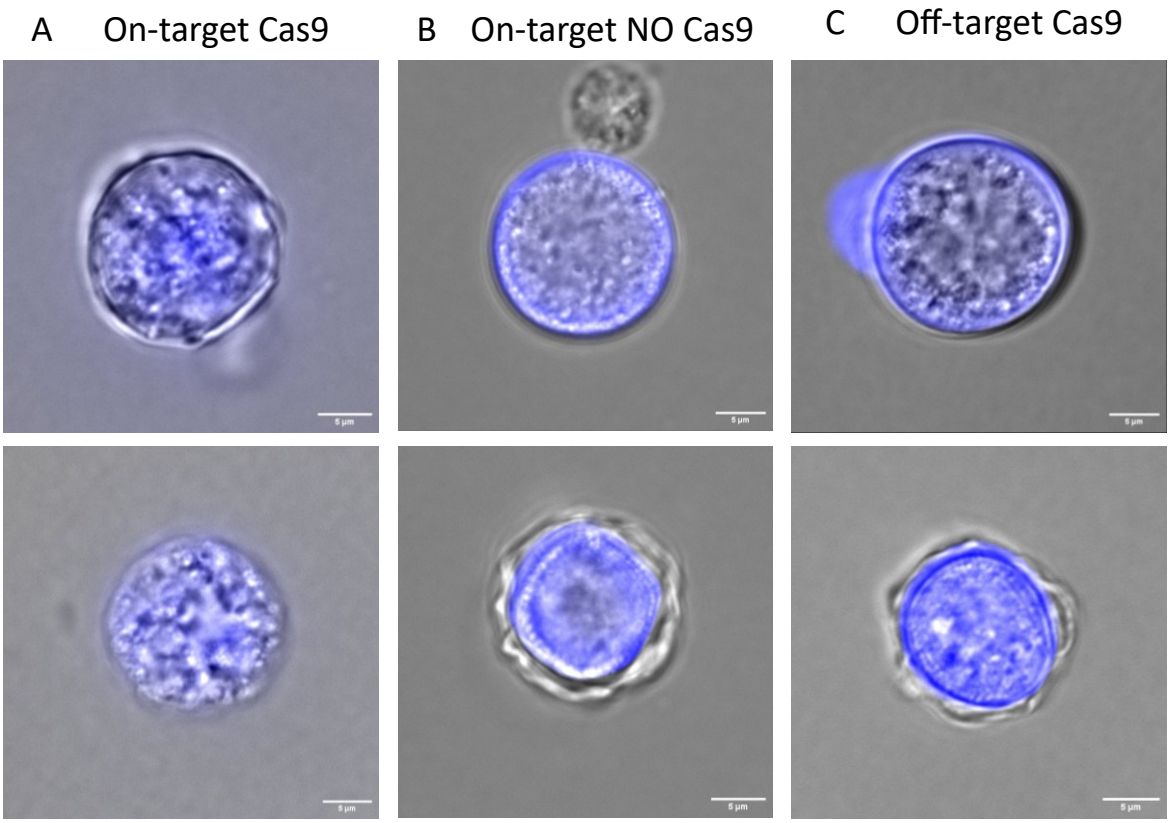

Figure 23. Confirmation of phenotype of cellulose synthase knockout strain. Cysts from different strains (in columns) stained with calcofluor (blue) merged with DIC channel. Strains used: A – on-target gRNA, Cas9 is expressed, B – on-target gRNA, Cas9 is not expressed, C – off-target gRNA, Cas9 is expressed.

5.5 ABC transporter antibodies

Polyclonal antibodies for the ABC transporter were prepared with recombinant antigen and immunisation of a rat. A segment of the ABC transporter was selected as antigen (Figure 24).

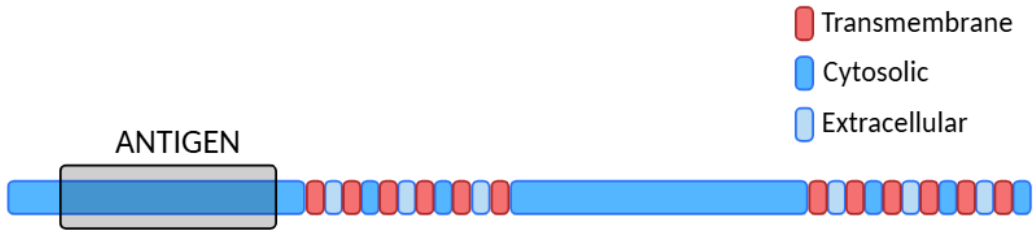

Figure 24. Schematic representation of the ABC transporter and the segment used as antigen for polyclonal antibodies. Created in BioRender.

5.5.1 Antigen isolation

As a first attempt to isolate antigen was used the histidine tag affinity to the Ni-NTA column. All the fractions were examined using SDS-PAGE and western blot. A western blot analysis was executed to specifically visualize the antigen in each fraction. The antigen was expressed in *E. coli* and bound to the column, however, we observed no signal on the western blot in the bacterial lysate fractions (wells IN, Figure 25). On the SDS-PAGE gel (Figure 25, panel A), the elution fractions E and I are clear of any excess protein from *E. coli*, although no protein including the antigen is visible. The antigen was present only in the first four elution fractions (D), where contamination of bacterial proteins is still present and only in inadequate amount for immunisation.

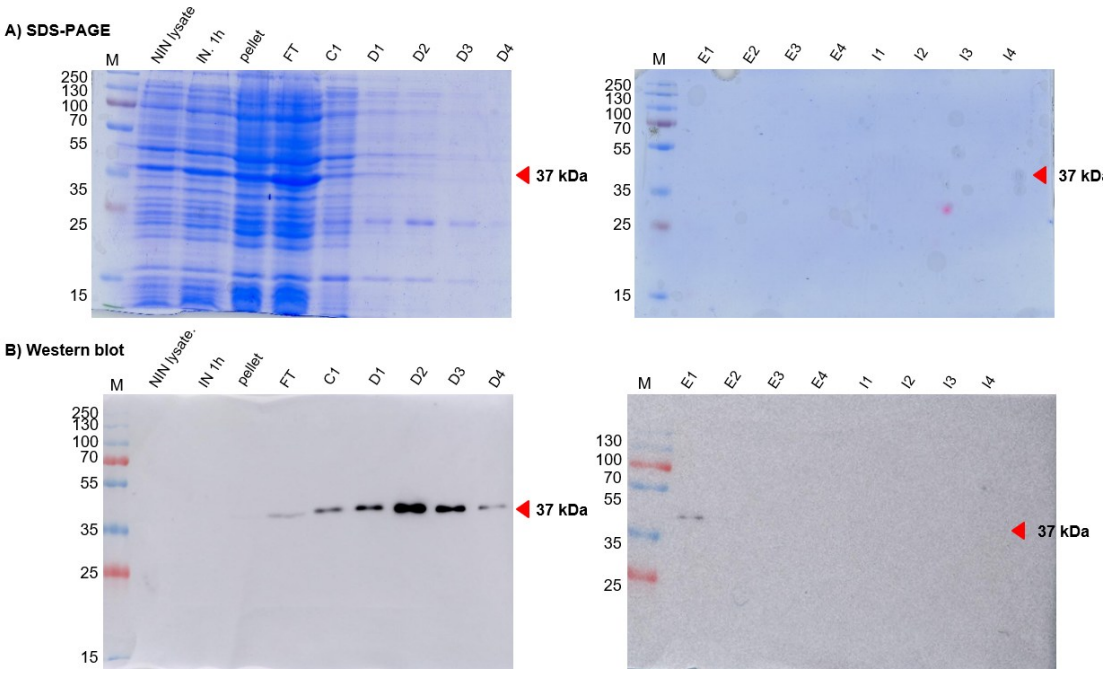

Figure 25. Ni-NTA purification of His-tagged antigen. **A)** SDS-PAGE gel stained with Coomasie Blue, each well with the next step of purification protocol. **B)** Western blot of the same fractions as in A. Wells: NIN lysate – bacterial culture before addition of IPTG, IN lysate 1h – bacterial culture 1 hour after IPTG addition, pellet – raw lysate, FT – unbound flow through, C1, D1-4 – wash fractions, E1-4, I1-4 – elution fractions.

Isolation of the antigen from inclusions was eventually carried out to upscale the experiment. The antigen gene was cloned into a new vector and the induction of expression was carried out in the same conditions and the inclusions of antigen were isolated (Figure 26). Antigen recovered from protein inclusions was used for immunisation of rat.

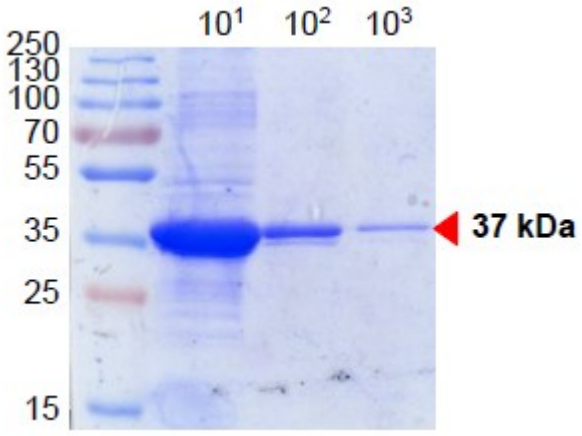

Figure 26. Isolation of antigen from protein inclusions. On SDS-PAGE gel 10-fold dilution of antigen protein sample.

5.5.2 Test of function

The specificity of the antibodies was tested on full cell lysate of the wild type *A. castellanii* and modified strains, the knockout strain and overexpressing strain. The ABC transporter is located in the cytoplasmic membrane, therefore, membrane fractions were also tested (Figure 27). The ABC transporter antibody shows only unspecific signal at 65 kDa, which does not correspond with the ABC transporter, that is the molecular weight of 158 kDa. The control, which is the isolated antigen, showed a positive result (Figure 27, panel A). One of the possible explanations for the absence of the antibody signal was low expression of the ABC transporter in normal state. That is why *A. castellanii* cells, which were incubated with PEITEC, a known drug to induce higher level of expression of the ABC transporter (Ženíšková et al., 2025), were tested by western blot analysis. In addition, considering that a 158 kDa protein migrates in the gel with difficulty, the stacking gel was included for the blotting (Figure 27, panel B). However, the signal showed no difference from the previous experiment. An antibody clean-up was carried out due to the unspecific signal of the antibodies. A full lysate of *A. castellanii* wild type stain was used to test the antibodies after clean-up., which again showed no signal in the cell lysate (Figure 27, panel C).

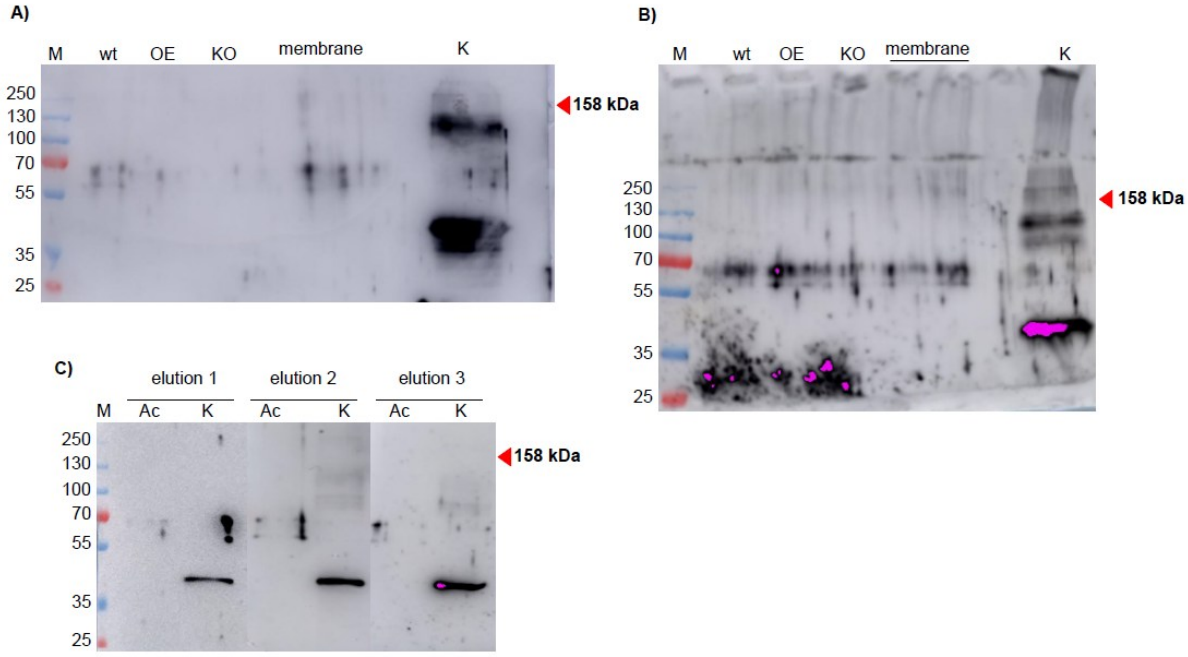

Figure 27. *A. castellanii* western blot analysis using antibody against ABC transporter. **A), B)** Full cell lysates of wild type strain (wt), ABC transporter overexpressing strain (OE) and ABC transporter single knockout strain (KO), wild type membrane fraction (membrane) and the isolated antigen sample as control (K) has been used. On blot B) all cell strains were first incubated with 6,25 uM PEITEC for 24 hours before lysis. **C)** The antibody clean-up test. All three elutions from clean-up were tested on wild type strain lysate (Ac) and as control (K) the isolated antigen has been used.

6 Disscusion

Despite many advances in treatment of infectious diseases, the emergence of resistance to chemotherapeutics and antibiotics often remains untackled. It leads to failure of treatment and, consequently, an increasing mortality. Resistance is an evolutionary process, and it is to some extent unpreventable, however, there are approaches to manage or impede the occurrence of resistance. Frequent and negligent use of drugs drives the emergence of resistance and suspends drugs from clinical use, therefore, it is recommended to use multiple drugs to treat a single infection. There are multiple mechanisms, with which the organisms can become resistant to a drug. Either via preventing the drug from influencing cellular processes, or via changing the targeted pathway on a molecular level, hence averting its toxic effect. A ubiquitous mechanism of resistance is the expression of multi drug resistance efflux pumps, which are responsible for the efflux of substances from the cell. This phenomenon was first described in bacteria, although, it also affects cancer cells and protozoan and viral infections. There are plenty of transporters that are responsible for MDR, one of them being the ABC transporters. In mammals in physiological condition the ABC transporters are utilized in cellular processing and transport of various bioactive molecules. Their adaptability to almost any substrate causes the drug resistant phenotype.

An unexpected result was obtained in the proteomic analysis of oxidative stress response of *A. castellanii* conducted by Ženíšková et al., 2025. The ABC type 2 transporter, ACA1_352460, was identified as significantly upregulated in the presence of PEITC and rotenone. It was reported that the expression of ABC transporters is induced by oxidative stress, either as a detoxification mechanism, or when they are accidentally affected (Yuan et al., 2022). Regardless, these results draw attention to its role in detoxification pathways in *A. castellanii* and its potential involvement in drug resistance. It is presumed to partake in cell efflux, which suggests its localisation in the cytoplasmic membrane. In order to assess its cellular localisation, the ABC transporter tagged with GFP was expressed in *A. castellanii.* Indeed, it was confirmed that the protein is localised in the cytoplasmic membrane as expected and the ABC transporter is a functional protein.

To further analyse the presence of the ABC transporter in stress conditions, we selected an additional drug to expose *A. castellanii* to a different type of stress. It was decided that amphotericin B was a viable option for a control drug, since it does not induce oxidative stress as its primary mechanism of action. Furthermore, amphotericin B is a commonly used drug to treat fungal and parasitic infections, including acanthamoeba infections (Cui & Zhao, 2024; Jariyapan et al., 2025; Reddy et al., 2022). Its primary role was only recently confirmed using an amphotericin B-silver conjugate, which revealed its fast disintegration of biological membranes and intracellular structures in fungal cells (Janik et al., 2023). In addition, it was proposed to interact with MDR transporters in *A. castellanii* (Taravaud et al., 2017).

We decided to observe changes of the ABC transporter on a transcription level, when exposed to certain stress conditions, namely amphotericin B, PEITC, rotenone. However, the results were partly contradictory with the proteomic analysis in Ženíšková et al., 2025, suggesting that the regulation occurs most likely posttranslationally. Similar effect was observed in *Naegleria gruberi* and *Naegleria fowlerii* (Arbon et al., 2020; Ženíšková et al., 2022). The protein levels showed significant changes, however, the transcription analysis revealed very dissimilar results in most transcripts, which represents a strong posttranslation regulation and the importance of examination of multiple regulatory levels (Arbon et al., 2020). On the other hand, in Grechnikova et al. (2022), a proteomic and transcriptomic analysis of *A. castellanii* iron homeostasis the expression levels correlate with the protein llevels,and no evident regulation was observed. Overall, this suggest, that the regulation of protein production in *A. castellanii* is mostly on the gene levels, however, posttranslational regulation cannot be excluded. The biggest change was present in the PEITC treatment. This corresponds with the fact that ABC transporters interact with PEITC, which is mostly used in cancer treatment, where it was proposed to function as an inhibitor of MDR (Aras et al., 2013; Tseng et al., 2002). The treatments with rotenone and amphotericin B did not show any significant changes in gene expression. Rotenone was therefore excluded from further experiments. Further analysis of Amphotericin B, on the other hand, had potential to provide insights into MDR in *A. castellanii*.

A standard method to assess the changes in protein production in different conditions is via western blot using specific antibodies. Preparation of polyclonal antibodies for the ABC transporter was included as a part of the methodology in this thesis. An antigen, which consisted of a cytosolic domain sequence, was used for immunisation of a rat. The purified antibody showed a great affinity to the synthesised antigen, however, no binding to the ABC transporter was present in the *A. castellanii* cell lysate. This could be due to the localisation of the transporter in the cytoplasmic membrane and many transmembrane domains in the protein, causing a limited ability of the antibody to bind.

The preparation of a knockout of the ABC transporter was a significant part of this thesis. Gene knockouts are an important technique to properly assess the function of the gene of interest. In this case we intended to test the genetically modified strains in stress conditions and compare how absence or presence of the ABC transporter affects viability of *A. castellanii*. There is a number of methods of knocking out a gene. A common method to knock out a gene is via homologous recombination of a selection cassette into the genomic sequence of choice. This experiment was designed to remove the whole genomic sequence of the ABC transporter and exchange it for a selection cassette. Two different selection cassettes were used for the transfections to increase the probability of recombination into the genome and upscale the selection process with two antibiotics, to potentially facilitate a more frequent recombination.

The first withstanding issue of knockout by homologous recombination is the ploidy of *A. castellanii*. Its ploidy was determined to be approximately 25n (Byers, 1986) in an experiment where the yields of genomic DNA from *A. castellanii* were compared with its haploid genome. Since then, a number of studies explored *A. castellanii* genomic attributes, however, the results were unable to define with certainty the ploidy of this organism. In the chromosome assembly study conducted by Matthey-Doret et al. (2022), the gene content was separated into scaffolds, which shed light on the genomic organisation and revisited the *A. castellanii* ploidy issue. Furthermore, this study showcased differences in the Neff strain (Neff, 1957) and the C3 strain (Michel & Hauröder, 1997) of *A. castellanii*, which are the most commonly used laboratory strains of *A. castellanii*. These strains differed in genomic content and size, however, it had no influence on the genomic organisation (Matthey-Doret et al., 2022). So even though the differences are not surprising, it is important to take into consideration the deviation in laboratory strains, since we use a clinical isolate of *A. castellanii*, which could potentially differ from the annotated strains.

To uncover the ploidy in various isolates of *A. castellanii* a recent study evaluated the frequency of alleles. The analysis showed a dynamic state of ploidy number in chromosomes, which inclined to higher numbers of copies. The conclusion pointed towards an everchanging number of chromosomes in laboratory culture, which exhibited aneuploidy, diploidy and polyploidy (Colp & Archibald, 2025). This insinuates, that the undisclosed number of copies of the ABC transporter could generate an issue in the efficacy of knockout via homologous recombination.

Examinations of cassette integration revealed a successful recombination with the gene, however, some copies remained intact without change. Aside from the high number of copies of the ABC transporter gene, a possible explanation for the poor knockout results is, that during the antibiotic selection the cells store the transfected cassette in episomes and therefore do not need to incorporate the cassette into their genome and survive the selection. Due to the unexplainedly varying transfection efficacy, a double check technique would increase the possibility of a successful knockout. A similar issue was observed in preparation of the overexpressing strain, which showed resistance to selection antibiotics and a low number of fluorescent cells. However, the overexpressing strain showed a high expression level of the ABC transporter mRNA, meaning that the GFP tag either does not fold properly in *A. castellanii* or that its function is restricted.

The genotype and phenotype of the knockout strains showed contradictory data in the knockout strain transfected with a single cassette and the strain transfected with two cassettes. It was expected that the ABC transporter expression with more transfected cassettes would decrease. But contrary to these expectations, the expression of the ABC transporter in the double transfected strain increased. Given the function of the ABC transporter, this could be a consequence of the stress induced by the presence of two selection antibiotics or compensatory upregulation.

Compensatory upregulation is a common phenomenon in organisms with a lack of functional allele, and it is compensated by high expression of the functioning alleles (Schreiber et al., 2025; X. Xu et al., 2019). There are reports of phenotype compensation in full knockouts of *Plasmodium berghei*, which describes more severe phenotype after the application of RNA interference, which induces changes only on mRNA level unlike knockouts (Hentzschel et al., 2019). This case presents the compensation in the lack of the genomic sequence in contrast with functioning gene copies present, which somewhat simulates the situation in the ABC transporter knockout. However, most cases of compensatory upregulation reported were induced by paralogs of the knocked-out gene (Borges et al., 2024; Schreiber et al., 2025).

To assess the role of the ABC transporter in resistance to drugs, a half inhibitory assay was performed using the drugs amphotericin B and PEITC and comparing the IC50 of strains with different levels of the ABC transporter. After the establishment of the improper knockout strain, it became evident that there would be complications with the assessment. Despite the issues, we proceeded with measuring the IC50. The double knockout was excluded due to the inexplicable increase of expression of the ABC transporter.

In PEITC the IC50 values were, as expected, decreased in the knockout strain in comparison with the wild type strain, indicating involvement of the ABC transporter in detoxification of PEITC. An obstacle with these measurement results was revealed in the control strains. The control, which consisted of cells resistant to the selection antibiotics, showed decrease in IC50 in comparison with the wild type. This was also potentially reflected in the overexpressing strain, where no change in IC50 value was observed compared to the wild type, however, that contradicts the hypothesis that the ABC transporter is a universal efflux pump. In conclusion, the PEITC treatment influences the levels of the ABC transporter, and its efflux is probably facilitated by the ABC transporter.

Amphotericin B, on the other hand, showed contradictory results, indicating that this ABC transporter involvement in this detoxification pathway is unknown. Even though these results are not definite and this experiment needs to be repeated with a different approach, it possibly reveals a specificity of the ABC transporter. Amphotericin B resistance is, however, facilitated by MDR transporters in *A. castellanii* and *Leishmania donovani* (Purkait et al., 2012; Taravaud et al., 2017). A genomic study of drug-resistant *Candida auris* strain identified a spectrum of ABC transporters in relation to MDR emergence, which also highlighted the differentiation in biological functions of ABC transporters and their relevance in drug resistance (Wasi et al., 2019). This coincides with our findings of different regulation of drug detoxification.

Furthermore, *A. castellanii* showed time dependent resilience to amphotericin B *in vitro*. After four days the IC50 values increased and a growth similar to the control was observed at seven days after the incubation with amphotericin B (Taravaud et al., 2017). The addition of an ABC transporter inhibitor delayed the acquisition of resilience to amphotericin B. Nevertheless, these

observations do not influence the measuring of IC50 in our experiment, since the cells were incubated in amphotericin B for three days and since the wild type offered similar results. However, the establishment of resilience of the herein used wild type strain might serve as a reference for future experiments.

After evaluation of these results, we searched for more proteins, that might be involved in MDR of *A. castellanii*. There were 7 paralogs of ABC type 2 transporters annotated in AmoebaDB, six of which included a full sequence (ACA1_114430, ACA1_192330, ACA1_0464460, ACA1_027020, ACA1_026990, ACA1_352460), with three of them also being included in the proteomic analysis (Ženíšková et al., 2025) and upregulated in PEITC. The ABC transporter family is usually represented by multiple proteins in the genome (Sheps et al., 2004), which is also the case in *A. castellanii*. Therefore, it is possible that knocking out one ABC transporter might not show any phenotype or change in susceptibility to drugs, due to compensation by the remaining ABC transporters, which was described in *Aedes aegypti* ABC transporter knockout (Pacheco et al., 2025). By this logic, it would be ideal to knockout more ABC transporters to induce the drug susceptible phenotype, however, a different knockout method would be necessary for that.

During the making of this thesis, a methodology for CRISPR Cas9 in *A. castellanii* was published (Philippe et al., 2024). It provided a detailed methodology for a robust cleavage of genes, which was well functional for *A. castellanii*, despite the difficulty of determining the number of gene copies. This method represents a promising perspective for future research, since reverse genetics is essential to demonstrate the function of the gene of interest and previously application of genetic tools was not possible in *A. castellanii*. The successful implementation of CRISPR Cas9 as a routine method advanced the research of *A. castellanii* in our laboratory. It was possible to produce a control knockout to determine, whether this method is suitable and assess troubleshooting. In Bisio et al. (2023), a cellulose synthase gene was subjected to a knockout. Considering the estimated 25n ploidy of *A. castellanii*, a knockout requires a robust method to cleave all the copies and preclude homologous recombination of the wild type copies.

As a preliminary experiment, the knockout of the cellulose synthase is a convenient candidate due to its confirmed function in cyst formation, specifically formation of the endocyst (Aqeel et al., 2013; Moon et al., 2014). Therefore, the phenotyping procedure is simple, it involves encystation and microscopic observation of the cyst wall malformation. The ABC transporter knockout was being prepared in parallel with the cellulose synthase, however, in hindsight the primer design was not suitable for Gibson cloning, and therefore the ABC transporter knockout was postponed.

Although further assessment of the role of the ABC transporter and transporters involved MDR in *A. castellanii* is needed. The current knowledge of MDR in *A. castellanii* is limited and requires more wide-scale studies to detect other involved components. Resistance to treatment is often managed by multiple proteins or molecules and cannot be explained by a single protein.

Considering that antifungal drugs are commonly used to treat *A. castellanii* infections, studies of MDR in fungal pathogens might provide insight into possible mechanisms of MDR or drug interactions in *A. castellanii*.

7 Conclusion

ABC transporters are ubiquitous proteins participating in the transport of various molecules and many cellular processes, however, their adaptability to substrates is the cause of emergence of resistance to treatment. In a recently published paper, the ABC transporter in *A. castellanii* was identified as an actor in the response to oxidative stress conditions. The aim of this thesis was to further describe the role of this ABC transporter in drug resistance utilizing genetically modified strains.

The first goal of this thesis was to identify the localisation of identified ABC transporter in the cell via overexpression of GFP-tagged protein. The cytoplasmic membrane localisation corresponds to other characterised ABC transporters and further suggests the function as a cellular efflux pump. Additionally, the expression levels of the ABC transporter were measured in three types of stress conditions. However, only one substance, PEITC, was proven to influence the ABC transporter expression. This implied a substrate specificity of the ABC transporter.

Observation of protein levels in different conditions is possible with the use of antibodies. One of the experiments aimed to prepare specific polyclonal antibodies against the identified ABC transporter. However, the antibodies were not specific when applied on *A. castellanii* lysate, and therefore we were not able to use them for any experiments.

The main premise was that the ABC transporter induces a drug-resistant phenotype in *A. castellanii*, which can be examined by preparation of the *ABC transporter* gene knockout strain and exposing it to the toxic conditions. Herein the preparation of knockout was achieved only partially with some of the wild type alleles still present in the genome. The probable reasoning for this is the high number of alleles, which caused insufficient recombination of the knockout cassette and generated issues for phenotyping. Similar to previous observations, changes were only present in PEITC treatment, which showed a decrease in viability of the ABC transporter knockout strain. This suggests that the ABC transporter interacts with PEITC, and that it is included in its detoxification pathways. In addition, on the basis of the inconclusive results in amphotericin B treatment, we can speculate that other ABC transporters are involved in the detoxification and therefore are substrate specific.

In the final parts of the making of this thesis we initiated efforts to produce a knockout via CRISPR Cas9. We were unfortunately only able to provide preliminary experiments for this method, however, the knockout of cellulose synthase was successful and serves as a reference for future experiments in the ABC transporter project. The CRISPR Cas9 is an adequate method for knocking out additional orthologs of the ABC transporter in one experiment and therefore establish a synergy of these proteins in drug resistance.

Overall, the role of the ABC transporter was only partially characterized, due to the unsuccessful knockout generation, which affected the experimental flow of this thesis. Nevertheless, the data

acquired show a plausible direction for the ABC transporter project and most importantly a feasible knockout method was established. For future studies of MDR in *A. castellanii,* it is necessary to properly identify the ABC transporters involved and their impact on the treatment, which is currently in clinical use. If MDR is proved to be a significant actor in drug resistance in *A. castellanii*, it could implement the administration of treatment with inhibitors of ABC transporters, drugs that have already been approved, to increase the efficacy of chemotherapeutics.

8 References

*review

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