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Characterization of Ato Family Transporters in the Fungal Pathogen Cryptococcus Neoformans

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CHARACTERIZATION OF ATO FAMILY TRANSPORTERS IN THE FUNGAL PATHOGEN *CRYPTOCOCCUS NEOFORMANS*

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ABSTRACT

Fungal pathogens are a significant threat to public health as they are becoming increasingly common and more resistant to treatment. *Cryptococcus neoformans* contributes greatly to this threat annually by causing an estimated 278,000 cases of cryptococcal meningitis resulting in approximately 181,000 deaths globally according to the CDC. *C. neoformans* is ubiquitous across most of the globe and can be found in such places as in trees or soil. Exposure to this fungus is especially dangerous to individuals who are immunocompromised or immunosuppressed. In these cases, inhalation of spores can lead to infection in the lungs. Once in the lungs, *C. neoformans* is often engulfed by alveolar macrophages intending to destroy the fungus but is often thwarted by pathogenic regulation of the macrophage peroxisome. This allows *C. neoformans* to travel through the blood brain barrier and infect the brain, leading to deadly cryptococcal meningitis. In shifting from the soil to lung tissue, several metabolic changes occur which allow the fungus to thrive on alternative carbon sources, such as acetate, due to the minimal levels of glucose found in early stages of infection. Acetate needs to be transported across the plasma membrane for activation to acetyl-CoA and utilization in a number of metabolic pathways. Many fungi maintain acetate transporters, many of which belong to the ATO (Ammonia Transport Out) family of transporters. In *C. neoformans*, the three ATO family members are *ATO1* (CNAG_05678), *ATO2* (CNAG_05266), and *ATO3* (CNAG_04787), which have yet to be characterized. In this study, we examine the phenotypes of single,

double, and triple mutants of the *C. neoformans ATO* genes to begin to elucidate their functions and possible role(s) in virulence.

DEDICATION

Hoc opus dedico Iesu Christo, Regi Creationis, soli gloriae.

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CHAPTER ONE

A REVIEW OF VIRULENCE, METABOLISM, AND TRANSPORT IN CRYPTOCOCCUS NEOFORMANS AND OTHER PATHOGENIC FUNGI

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Importance of Virulence in *Cryptococcus neoformans*

The fungal pathogen *Cryptococcus neoformans* causes a substantial number of infections and deaths yearly across the globe. As of 2017, C. *neoformans* annually causes an estimated 278,000 cases of cryptococcal meningitis resulting in approximately 181,000 deaths worldwide according to the CDC (Rajasingham et al., 2017). Most of these cases are found in Sub Saharan Africa and Asia, but cases can be found on six of the seven continents (Rajasingham et al., 2017). C. *neoformans* is particularly pervasive among immune compromised individuals such as those who are living with HIV/AIDS (Rajasingham et al., 2017).

Additionally, few antifungals have been capable of consistently treating cryptococcal meningitis (Vanreppelen et al., 2023). One common treatment option used is fluconazole, which was patented by Pfizer in the 1980s and requires a standard treatment period of six months (Perfect et al., 2010). The current WHO recommendation for initial treatment in HIV positive patients is amphotericin B in combination with other antifungals (WHO guidelines for diagnosing, preventing and managing cryptococcal disease among

adults, adolescents and children living with HIV, 2022). It is important to keep in mind that no impactful antifungal treatments have been introduced within the last thirty years (Vanreppelen et al., 2023). Given the emergence of outbreaks and the potential for antifungal resistance of C. *neoformans*, this organism is a critical priority for research and public health as stated by the WHO shown in **Figure 1** (WHO Fungal Priority Pathogens List to Guide Research, Development and Public Health Action).

Pathogenic Life cycle of *Cryptococcus neoformans*

This fungus is usually found in soil or certain kinds of trees, and in rarer cases bird guano (Sorrell and Ellis, 1997; Emmons, 1955). Infection is typically initiated by inhalation of spores into the lungs (Chen et al., 2013). Individuals with healthy immune systems typically clear early pulmonary infections, but immunocompromised individuals often face great difficulty. Even cases where an apparently healthy individual faces an established infection, many individuals have been found to have defects in macrophage stimulating antibodies (Maziarz and Perfect, 2016; Chen et al., 2014). Once in the lungs, C. *neoformans* undergoes a series of metabolic adaptations to maintain a foothold in its new environment (Hu et al., 2008). The invasion often progresses when the fungus is phagocytosed by alveolar macrophages (Feldmesser et al., 2000). C. *neoformans* survives the phagocytosis via peroxisome regulation and can cross the blood brain barrier (BBB) via the "Trojan Horse" model which leads to cryptococcal meningitis (Charlier et al., 2009). The life cycle of C. *neoformans* is displayed in **Figure 2** to encapsulate each of these steps.

***C. neoformans* virulence and response**

C. neoformans has a variety of virulence factors, some of which are unique to the organism, that allow it to invade a human host and evade destruction by the immune system. C. *neoformans* is thought to maintain such a high prevalence in humans due to its complex capsule formation, melanin production, and thermotolerance (Taylor-Smith and May, 2016).

C. *neoformans* produces a capsule with a unique composition alongside its cell wall (**Figure 3)** (Casadevall et al., 2019). The capsule is predominantly made of glucuronoxylomannan (GXM) (90%–95%) and glucuronoxylomannogalactan (GXMGal) (5%–8%) (Casadevall et al., 2019, Wang et al, 2018). These polysaccharides allow for substantial immune evasion through reduced immune responsiveness, reduced phagocytosis by macrophages, and increased survival within macrophages (Wang et al.,1995). GXM and GXMGal are also recorded to offer protection depending on what type of immune cell is confronting *C. neoformans* (Vecchiarelli et al., 2013).

Another foundational component of the cell envelope is melanin (Nosanchuk and Casadevall, 2006). Melanin is part of the cell wall, which offers protection against environmental stressors, host immune responses, and antifungals (Nosanchuk and Casadevall, 2006, Pukkila-Worley et al., 2005). Melanin is thought to be particularly useful in protecting cells from foreign enzymes that degrade the cell wall because? (Rosas and Casadevall, 2001).

For an organism to invade a mammalian host, it must be able to survive an internal body temperature of approximately 37°C (Casadevall, 2005). *C. neoformans* is well known

to be able to survive at this temperature (Casadevall, 2005). In fact, some species of *Cryptococcus* have been found to survive temperatures up to 40°C (Perfect, 2005). The ability to survive relatively high temperatures is often considered to be the most critical virulence factor due to the aforementioned reason of host environment survival. Various strains of C. *neoformans*, namely var. *gattii*, maintain several known pathogenic traits, including melanin and capsule formation, macrophage survival, and metabolic flexibility, but these organisms are not known human pathogens because of temp? (Martinez et al., 2001).

Interactions between *C. neoformans* and immune cells can result in many different outcomes, many of which can help disseminate the fungus rather than eliminate it (**Figure 4**). One of the characteristics that makes C. *neoformans* so pervasive is its ability to survive phagocytosis and invade vulnerable tissues (Charlier et al., 2009). Once engulfed, C. *neoformans* has been found to cause a disruption in the phagosome membrane and an increase in relative phagosomal pH (Tucker and Casadevall, 2001). This mechanism allows for the continued survival of the fungus while it resides within the macrophage. Because of this, C. *neoformans* evades destruction and while generating nutrients for subsistence (Tucker and Casadevall, 2002). Residency in host macrophages also shelters the pathogne from other immune cells and intercellular environmental stressors (Mansour et al., 2004, Chaturvedi et al., 1996). As macrophages move between tissues, C. *neoformans* is brought with them. At a certain point, C. *neoformans* can escape the phagosome, and the fungus is released into an area of the host that would otherwise be inacccessible (Charlier et al.,

2009). This includes the central nervous system, where the fungi can cause cryptococcal meningitis (Charlier et al., 2009).

Thre are metabolic changes that occur in C. *neoformans* during virulence. Glycolysis, gluconeogenesis, β-oxidation, the citric acid cycle and glyoxylate shunt all play a significant role in *C. neoformans* virulence (Cherniak et al., 1998). Expression of several glycolytic genes, including phosphofructokinase, hexokinase, and enolase, has been found to be increased in murine lung tissues (Hu et al., 2008). When genes encoding pyruvate kinase (*pyk1*Δ) and hexose kinase I and II (*hxk1*Δ*/hxk2*Δ) were deleted, the resulting mutants displayed attenuated virulence in mice (Price et al., 2011). This evidence suggests that glycolysis plays an important role in virulence. Fatty acid β-oxidation is another area of interest due to its secondary metabolite production (Hiltunen and Qin et al., 2000). When the genes encoding peroxisomal β-oxidation enzymes such as Mfe2 and Had1 were deleted, C. *neoformans* had a reduced ability to grow on fatty acids, truncated ability to develop a capsule, and limited ability to invade the brain (Kretschmer et al., 2012). There was an additive effect when multiple β-oxidation proteins were knocked out, with doubling survival time and reducing brain invasion by half in mice compared to the wildtype (Kretschmer et al., 2012). Arguably, both β-oxidation and glycolysis are necessary for virulence and continue to play a vital role in our understanding of the pathogen.

Attempts to control *Cryptococcus neoformans* pathogenicity

The primary method used to combat fungal infections depends on the location of infection, but typically consist of treatment with azoles, polyenes, or echinocandins shown

in **Figure 5** (Lee et al., 2021). Azoles, usually in the form of fluconazole, work by disrupting ergosterol synthesis utilized in the cell membrane (**Figure 6**) (Perfect, 2017, Robbins et al., 2016). This leads to a structural weakness in the membrane as well as an accumulation of toxic intermediates (Perfect, 2017, Robbins et al., 2016). Polyenes, often amphotericin B, also disrupt the cell membrane but do so by acting as a sink for sterols ("sterol sponge") (Anderson et al., 2014). Once again, membrane structure is weakened, allowing for leakage of intracellular components (**Figure 6**) (Anderson et al., 2014). Echinocandins, the last class of drug commonly used for treatment, bind certain components of the cell wall leading to a loss of homeostasis (**Figure 6**) (Letscher-Bru and Herbrecht, 2003). This class of drugs is generally well liked by researchers due to its ability to target fungi with low host cell cytotoxicity. Caspofungin is an example of a commonly used echinocandin, but unfortunately it is relatively ineffective in treating cryptococcal meningitis (Perfect, 2017). The standard treatment of C. *neoformans* often involves a combination of several of drugs delivered for several months or even longer (Perfect, 2010).

Vaccines have also been developed with a goal to tame fungal pathogens (Robbins et al., 2016). Though this avenue is generally considered ineffective to date in C. *neoformans*, some promising developments have been made in an antigen-based vaccine *for Candida albicans* (Armstrong-James et al., 2017). With this in mind, we see the importance of further research into this organism to find more versatile and effective treatments for cryptococcal meningitis.

Metabolic Flexibility of Pathogenic Fungi

C. *neoformans* and other fungi are capable of capitalizing upon many different carbon sources and nutrients for survival. This flexibility provides the opportunity to colonize new environments, including the human body. There are several conserved systems for recognition and regulation across fungi (Ries et al., 2018).

Glucose is the preferred carbon metabolite in fungi, therefore systems regulating its intake are well studied (**Figure 7)** (Ries et al., 2018). In *Saccharomyces cerevisiae*, *C. albicans*, *Aspergillus fumigatus*, and *C. neoformans*, a glucose receptor, typically Gpr1 or homolog, is used as a receptor in a signaling pathway that ultimately regulates cAMP responsive proteins as well as glycolysis which shows how ubiquitous this type of metabolic regulation is across fungi (Ries et al., 2018). In the absence of glucose, ATP is generated in other ways. This can take the form of alternative carbon source utilization, nitrogen switching, and even amino acid metabolism (Ries et al., 2018).

Alternative metabolic pathways like β-oxidation and the glyoxylate cycle often use acetate or lactate (Price et al., 2011) as ? for?. Acetate is imported and can be converted into acetyl-CoA by an acetyl-CoA synthetase (Acs) and utilized in the citric acid cycle, the glyoxylate cycle, or in gluconeogenesis (Hynes et al., 2011). Acs is particularly important as shown by an *ACS* knockout in *C. neoformans* being incapable of growing on acetate, glycerol, and ethanol as carbon sources and displays reduced virulence even though the mutant has no defects in common virulence factors (Hu et al., 2008).

Lactate is imported and usually converted to pyruvate by a lactate dehydrogenase which can also be used in the citric acid cycle to produce ATP (Guiard, 1985). Genes

related to lactate metabolism and other alternative carbon sources are upregulated in *C. albicans* in response to being phagocytosed by macrophages (Ene et al., 2012). Many other short chain monocarboxylates can be utilized in this way, as seen by higher expression of monocarboxylic acid transporters (Robellet et al., 2008), but these other fatty acids occur less frequently in host environments, leaving the importance of acetate and lactate apparent. Some alternative carbon sources like lactate and amino acids even promote resistance to common antifungals such as fluconazole and caspofungin in certain pathogenic fungi (Williams and Lorenz, 2020). It is clear that fungi can thrive on a wide range of substrates which are found broadly across different settings.

Acetate Utilization as a Carbon Source Alternative

In environments in which the preferred carbon source glucose is limited, C. *neoformans* and other fungi often utilize acetate, a two-carbon, short chain fatty acid, as a carbon source. It is hypothesized that acetate is preferred in *C. albicans* due to its short length and its ability to feed into the glyoxylate cycle as acetyl-CoA (Carman, 2008). This preference in *C. neoformans* is evident in SAGE data showing upregulation of genes involved in acetate utilization proteins. For example, the gene encoding Acs is upregulated(Hu et al., 2008). Acetate can also be utilized in cell signaling and environment when excreted as acetic acid (Řičicová et al., 2007). P. *lutzii* and P. *brasiliensis* both increase protein expression, especially proteins related to the glyoxylate cycle, when grown on acetate (Baeza et al., 2017). These enzymes allow these fungi to survive in the host environment. A proteomic profile study revealed similar increases in glyoxylate genes in

Candida glabrata, including isocitrate lyase (Icl1), malate synthase (Mls1), aconitase (Aco1) and citrate synthase (Cit1) (Chew et al., 2021). This leads to higher protein synthesis feeding into the glyoxylate cycle. *A. fumigatus* is also well known to utilize acetate in host invasion when glucose levels are minimal, particularly by upregulating FacA and FacB (Ries et al., 2021). This leads to the question: How does acetate enter into the cell?

Acetate transport is common across fungi

To obtain acetate for use in metabolism, C. *neoformans* and other fungi have transporters to facilitate movement across the plasma membrane. This is particularly important at low concentrations of acetate and at specific pH values (Wolfe, 2005). The yeast *S. cerevisiae* relies on Ady2p, an acetate permease, to import acetate into the cell (Paiva et al, 2004). *Yarrowia lipolytica*, a yeast commonly associated with food microbiomes, has a gene termed *GPR1* that is considered analogous to *ADY2P* (Augstein et al., 2003). Gpr1 was originally thought to play a role in glyoxylate enzyme repression but has been more recently shown to act as a sensor for acetic acid (Augstein et al., 2003) through regulation of proton pumps that mitigate environmental pH in the presence of acetic acid (Gentsch and Barth, 2005). *Aspergillus nidulans*, a relative of the pathogenic *A. fumigatus*, maintains expression of AcpA which is critical for transporting acetate and regulating pH (Sá-Pessoa et al., 2015). When pressured with an acetate pulse, A. *nidulans* fail to uptake radiolabeled acetate when the gene encoding AcpA was deleted (Sá-Pessoa et al., 2015). This finding was confounded when this same result was shown in early growth

stages compared to log phase growth (Sá-Pessoa et al., 2015). Similar studies have shown that there is a growth defect when grown on low acetate concentrations, particularly when pH is increased (Robellet et al., 2008). This information is of note as environmental pH is known to play an important role in phagocytosis (REFS). AcpA may also function as a low affinity transporter for similar monocarboxylates, which could expands the capacity for metabolic flexibility in fungi (Sá-Pessoa et al., 2015). Thematically, acetate usage is critical for survival in host environmental conditions. Other factors such as pH and temperature are also involved in this interaction. Another important fungal pathogen, *C. albicans*, uses a large family of acetate transporters, some of which transport acetate out of the cell, rather than into the cell (Danhof and Lorenz et al., 2015). Many acetate transporters, whether transporting in or out, fall into a related family referred to as the ATO family. This family of genes was termed ATO or Ammonia Transport Out because of their role in release of ammonium in *S. cerevisiae* (Řičicová et al., 2007).

ATO family transporters play a role in environmental regulation

ATO families are found in a variety of fungi, particularly pathogens, but what is their importance and why are they so frequently conserved? In *S. cerevisiae*, three transporters (Ato1p, Ato2p, and Ato3p) are proposed to play a role in acetate transportation and ammonia production (Palková et al., 2002). The localization of yeast Ato transporters has been shown in the plasma membrane using GFP tagging (Řičicová et al., 2007). In this same study, ammonia production was found to be associated with localization, and expression was increased in the presence of ammonia (Řičicová et al., 2007). Yeast likely uses its transporters to export ammonia for signaling to other colonies to cause a shift in

growth patterns in a sort of 'ammonia chain reaction'. (Řičicová et al., 2007). These transporters are observed to have an additive effect on ammonia production, as deletions of single transporters result in partial defects (Palková et al., 2002). Gpr1, which is analogous to Ady2p, has been shown to function as an acetate sensor rather than an acetate transporter (Augstein et al., 2003), suggesting that some putative acetate transporters are ammonia exporters or acetate sensors. These proteins allow fungi to regulate and respond to their environment as well as signal to other colonies. AcpA mutants in *A. nidulans* have growth defects that are exacerbated when pH is higher, but these defects are not likely related to ammonium efflux as has been seen in other organisms (Sá-Pessoa et al., 2015). When AcpA is missing, other proteins are thought to permissively compensate for it such as AcpB which maintains acetate transport in the mycelia, and JenA and JenB which are thought to be more promiscuous mono and dicarboxylic acid transporters (Sá-Pessoa et al., 2015).

C. albicans is a human fungal pathogen that is related to *C. neoformans*. One of these aspects is its own family of putative acetate or ammonia transporters (Vylkova et al., 2011). The genes encoding these transporters have been found to be significantly upregulated when phagocytosed by macrophage (Danhof and Lorenz et al., 2015). C. *albicans* requires certain Ato transporters to raise the environmental pH, likely indicating an expulsion of ammonia via these same transporters (Danhof and Lorenz et al., 2015). In relation to this alkalization, loss of specific Ato transporters substantially inhibits C. *albicans'* ability to damage and escape the macrophage phagosome (Danhof and Lorenz et al., 2015). In total, five C. *albicans* Ato transporters have been shown to contribute to

alkalinization when overexpressed (**Figure 8**) (Danhof and Lorenz et al., 2015). These transporters play a substantial role in pathology, particularly in relation to macrophage survival. The large number of genes related to acetate/ammonia transport in C. *albicans* is attributed to evolutionary pressure that? (Almeida et al., 2008)(Paiva et al., 2004). It has been hypothesized that some of these transporters have evolved to become sensors that indicate environmental changes to the cell (Priest and Lorenz, 2015). Ultimately, these discoveries in C. *albicans* can offer us insight into the function of this family of transporters in C. *neoformans*.

Three putative ATO family members with similar identity have been discovered in the *C. neoformans* genome but have yet to be fully characterized. Genes encoding two of these transporters (Ato1 and Ato2) have been found to have elevated expression in mouse lung tissue during early and onset infection (Hu et al., 2008). These tissues are known for their low levels of glucose, and for the presence of acetate (Garnnett et al., 2012). An *ato1Δ* mutant has further shown a growth defect when grown on acetate as the sole carbon source (Grace Kisirkoi and Kerry Smith, unpublished). In *Galleria mellonella* survival assays, both *ato1Δ* and *ato1ato2Δ* mutants resulted in a strong trend of virulence attenuation compared to wild type fungi (Grace Kisirkoi and Kerry Smith, unpublished). When this experiment was repeated in mice, only the *ato1ato2Δ* mutants was attenuated (Charles Specht, Grace Kisirkoi, Kerry Smith, and Stuart Levitz, unpublished). Surprisingly, the *ato1ato2Δ* mutant displayed several different phenotypes that the single *ato1Δ* or *ato2Δ* mutants did not, such a lack of thermotolerance and capsule formation. To confirm that the phenotype of the double mutant was the result of deletions of both the *ATO1* and *ATO2*

genes, and not the result of an additional unmarked alteration generated during the construction of the *ato1ato2Δ* mutant (Baltes et al., 2017), we generated new double and triple deletion mutations of the *ATO1*, *ATO2*, and *ATO3* genes via a CRISPR-Cas9 system.

Conclusion

C. neoformans remains a highly pervasive disease globally among other fungi. These fungi maintain the ability to thrive in nearly all environments, utilize many different carbon sources for metabolism and biosynthesis, and can survive on acetate as a sole carbon source via membrane transporters. Acetyl-CoA synthetase has been shown to be necessary for growth on acetate to maintain full virulence (Hu et al., 2008). In this review, we score a variety of virulence factors and metabolic mechanisms that allow for such versatility (Coelho et al., 2014) and have explored how and why fungi would utilize acetate. We also briefly discuss how other monocarboxylates often play a similar role. Lastly, we examined the intricacies of acetate transport in conjunction with its part in fungal virulence, as well as closely related functions such as ammonium export. Ultimately, we unify several areas of literature to construct a paradigm, understanding the complex mechanisms that make pathogenic fungi so invasive.

Understanding transporters and alternative metabolism in pathogenic fungi allows scientists to further understand how this subset of microorganisms survives in uncommon environments. Fungal metabolic flexibility involves a pathway of proteins, the first of which is most often a transporter. Transporters in some fungi have been shown to be the lynchpin of their survival in host phagosomes (Danhof and Lorenz, 2015). In other fungi,

transporters have been shown to allow the continuation of growth in areas lacking common metabolic resources (Paiva et al., 2004). Yet further, these transporters are used in signaling between colonies, directing growth shifts (Augustein et al., 2003). Lastly, similar transporters have been shown to regulate environmental pH (Carman, 2008). With this in mind, we aim to triangulate the discussion around the importance of transporters, particularly in regard to their role as potential drug targets.

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Figures

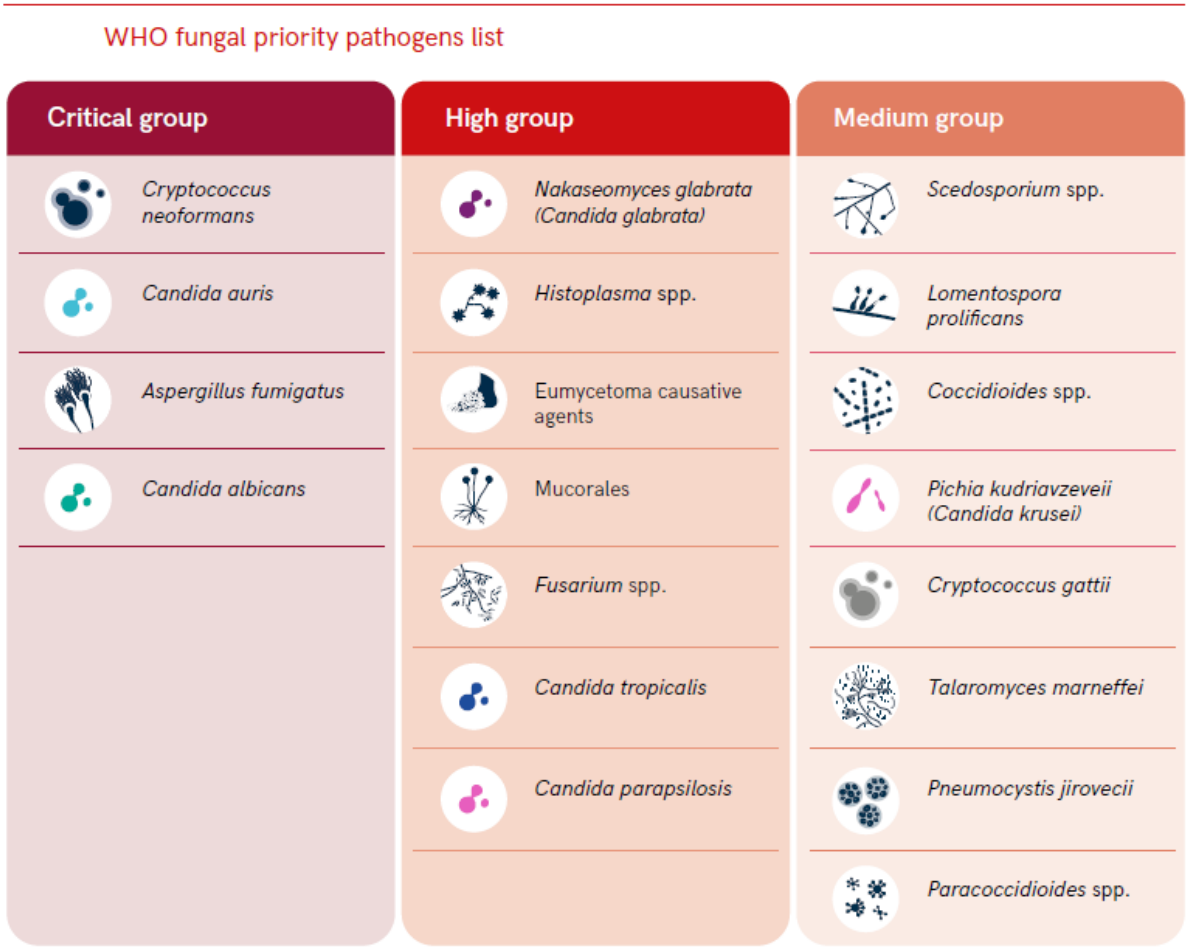

Figure 1.1: **WHO Fungal Priority Pathogens List.** *C. neoformans* is considered the highest priority fungal pathogen, belonging to the critical group, as seen in the WHO Fungal Priority Pathogens List to Guide Research, Development and Public Health.

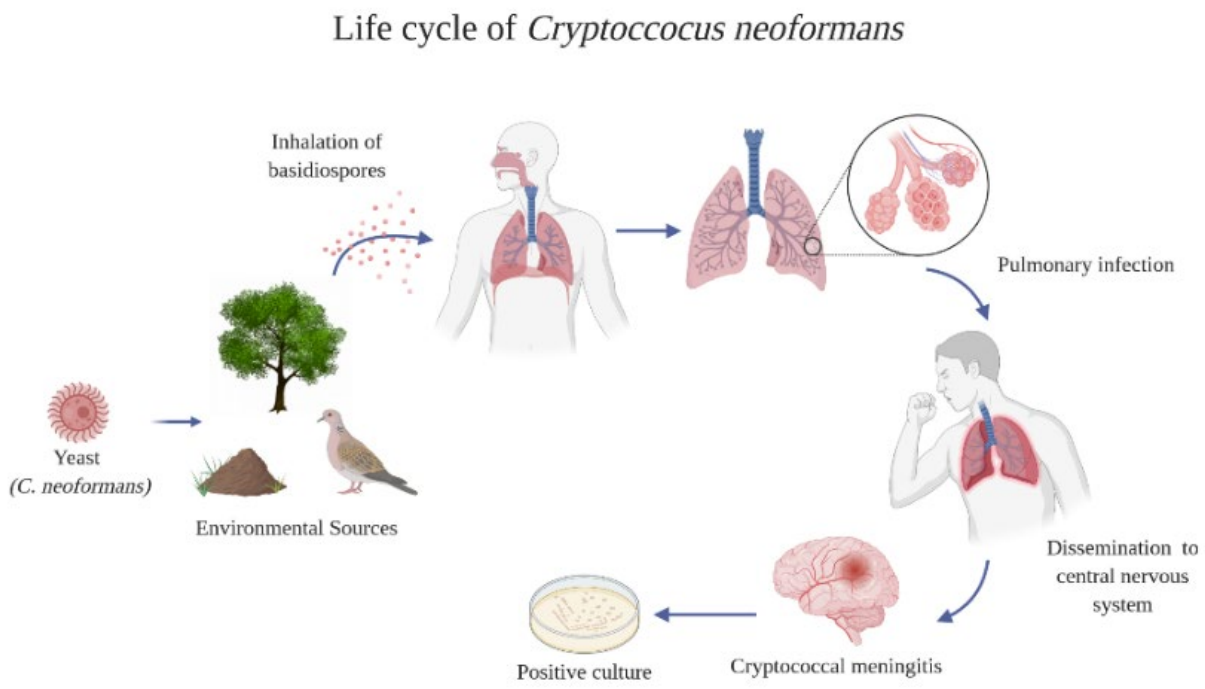

Figure 1.2: **Life Cycle of *C. neoformans*.** The *C. neoformans* pathogenic life cycle begins as an environmental fungus in soil and typically ends by causing cryptococcal meningitis. Dr. Perry Kezh in the Smith lab using Biorender.

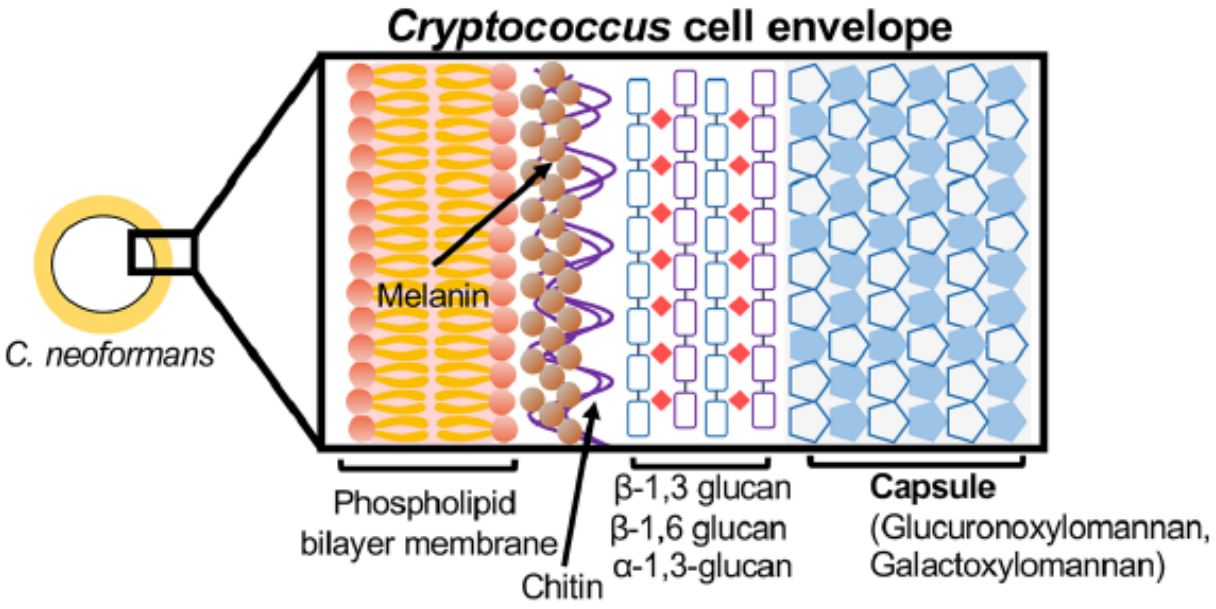

Figure 1.3: **Capsule components of *C. neoformans*.** The C. *neoformans* cell envelope components vary from other fungal pathogens, leading to a unique virulence factor. Of particular interest is melanin, glucan and mannan. This figure is from Bahn et al., 2020. We have either received permission from the publisher to use this figure, or it falls under open access.

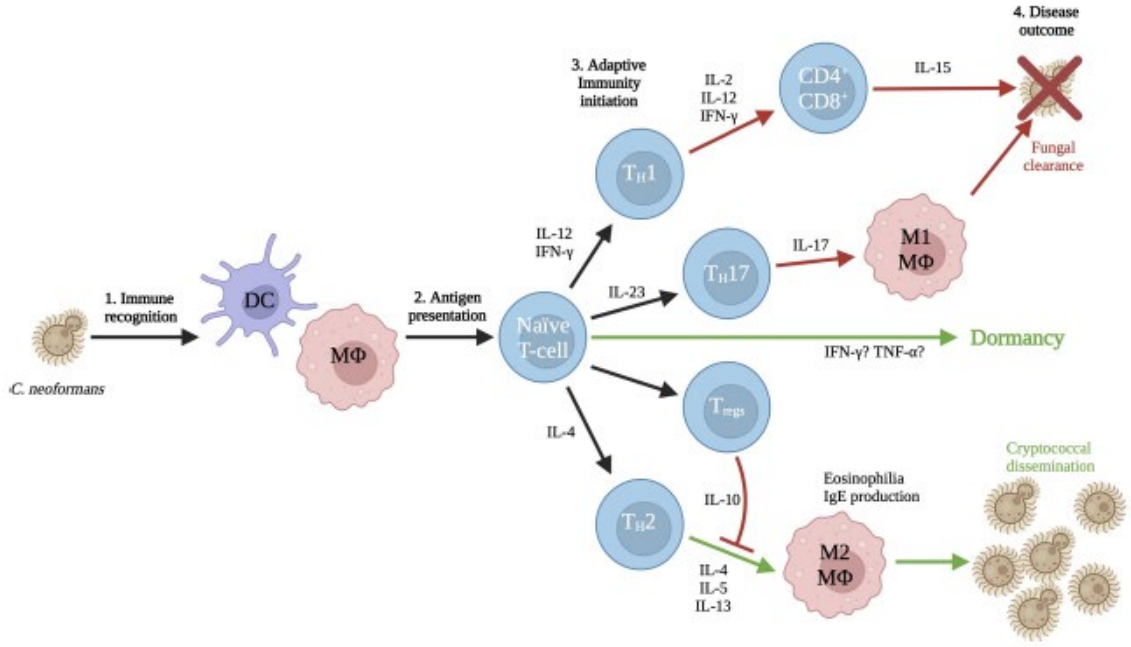

Figure 1.4: **Immune cell interactions with *C. neoformans***. Immune cell interactions with *C. neoformans* vary in outcome. The immunological pathways of interest involve T cell signaling that result in fungal dissemination. This figure is from Berguson et al., 2022. We have either received permission from the publisher to use this figure, or it falls under open access.

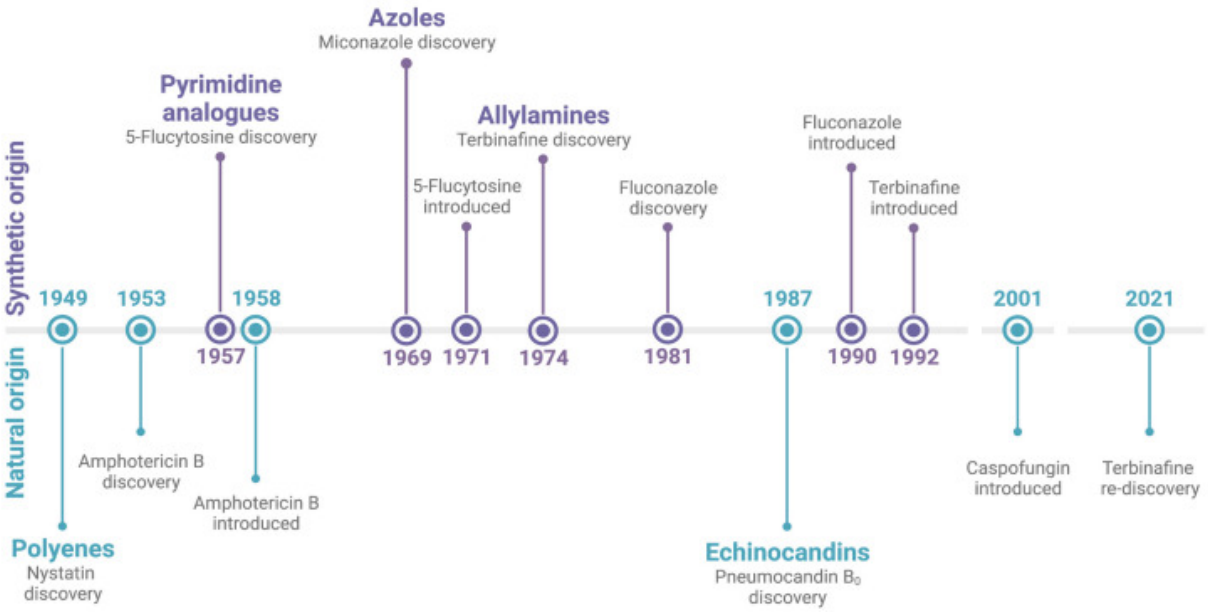

Figure 1.5: **Antifungal drug development**. Antifungal drugs have been developed since the 50's. Polyenes and Echinocandins, two of the most commonly used categories, were derived naturally from microorganisms. Azoles were developed in the 70's, with the most notable discovery being Flucanazole in 1981. This figure is from Vanreppelen et al., 2023. We have either received permission from the publisher to use this figure, or it falls under open access.

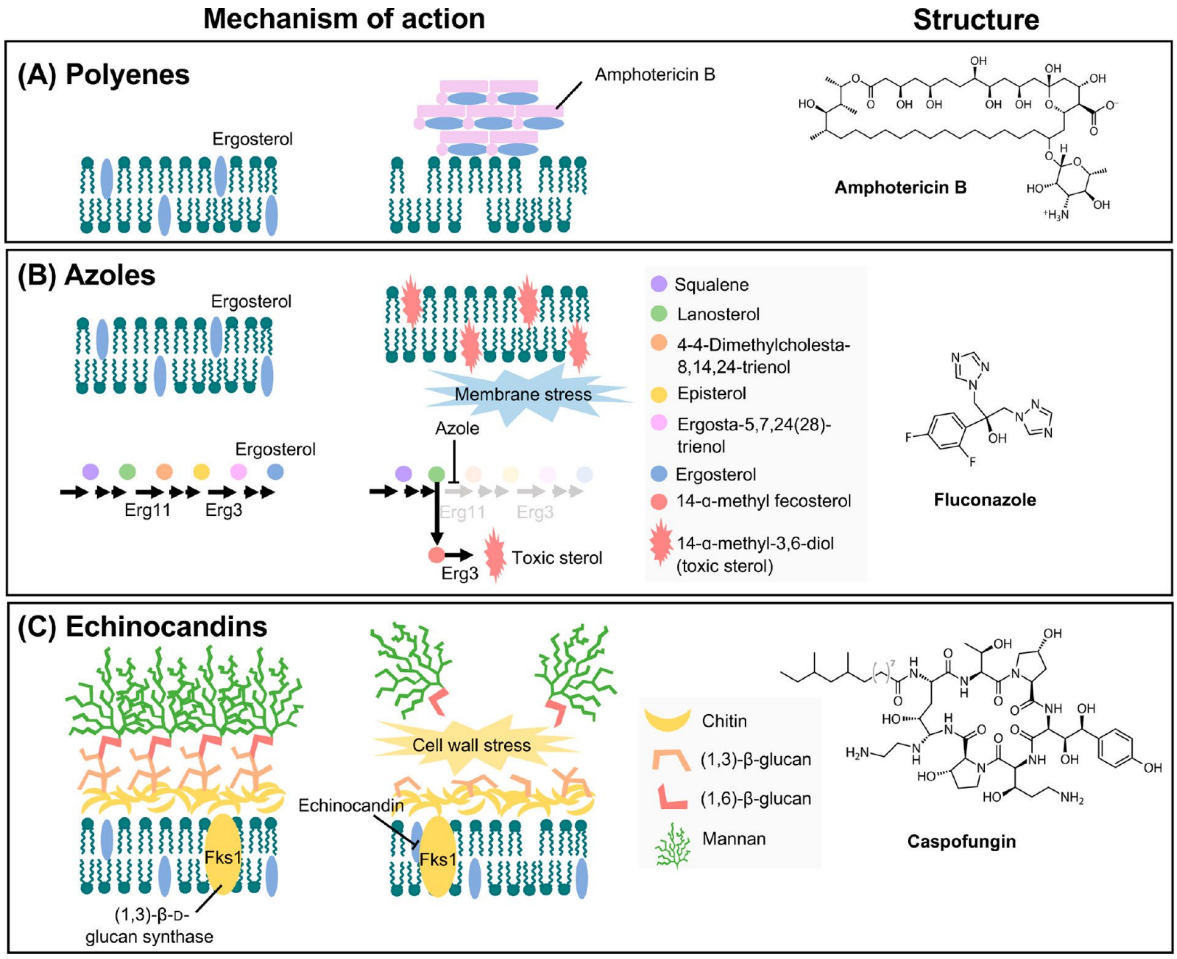

Figure 1.6: **Mechanisms of common antifungals**. Antifungal drugs operate using several different mechanisms, but most often involve disruption of the cellular envelope. Polyenes sequester ergosterol from the plasma membrane. Azoles disrupt ergosterol production and produce toxic intermediates. Echinocandins inhibit Fks1 which disrupts cell wall construction, creating a vulnerability to cell wall stress. This figure is from Lee et al., 2021. We have either received permission from the publisher to use this figure, or it falls under open access.

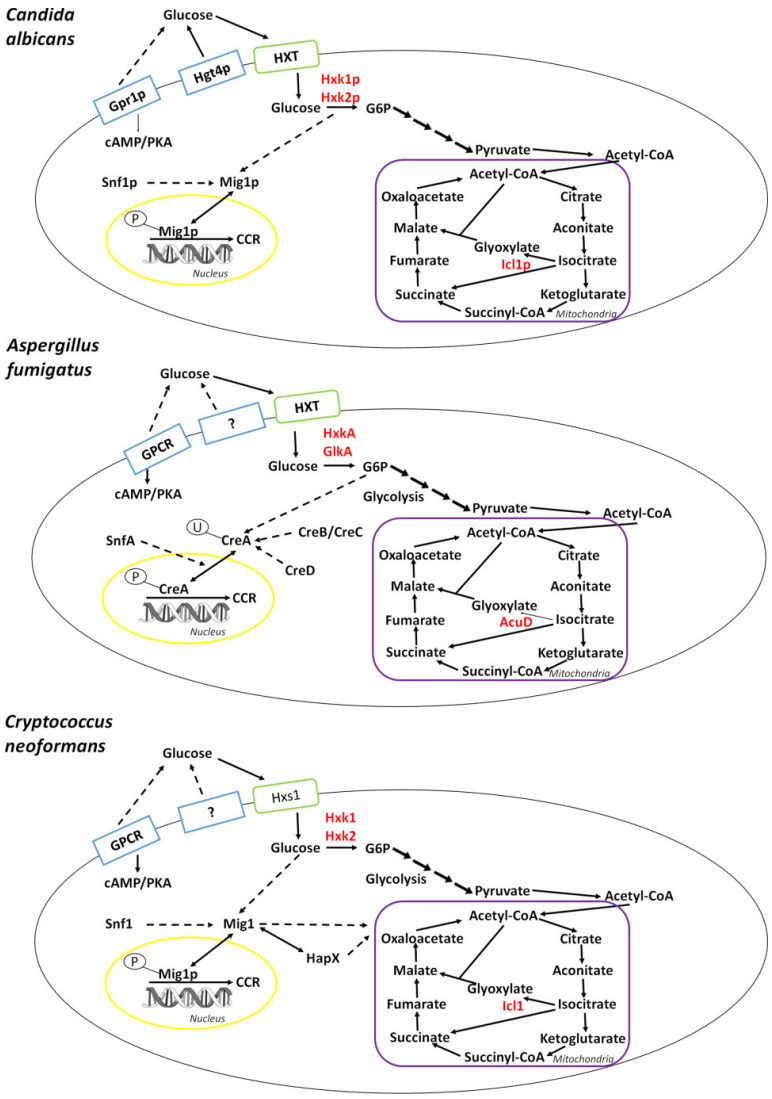

Figure 1.7: **Glucose metabolism of pathogenic fungi**. Glucose sensing, intake, and metabolism is shown in *C. albicans*, *A. fumigatus*, and *C. neoformans*. This figure was created by Ries et al 2018. (blue boxes: confirmed and putative sensors; green boxes: confirmed and putative transporters; red = metabolic enzymes, solid arrows = confirmed cellular processes, dashed arrows = cellular processes that are not elucidated; yellow =

nucleus; purple = mitochondria). We have either received permission from the publisher to use this figure, or it falls under open access.

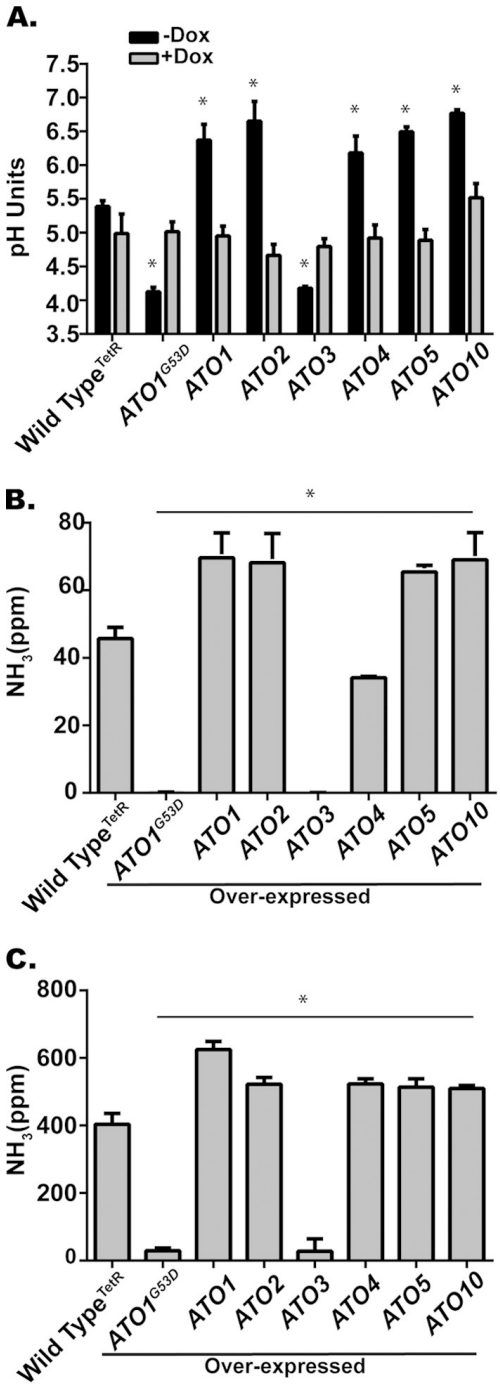

Figure 1.8: **Overexpression of ATO genes causes ammonia release in *C. ablicans*.** Overexpression of several ATO genes using a doxycycline repressible promoter in *C. albicans* resulted in a significant increase of ammonia release. This is thought to be

related to macrophage survival and escape as well as environmental control. This figure is from Danhof and Lorenz, 2015. We have either received permission from the publisher to use this figure, or it falls under open access.

CHAPTER TWO

INVESTIGATING THE ROLE OF ATO FAMILY TRANSPORTERS IN *CRYPTOCOCCUS NEOFORMANS*

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Abstract

The fungal pathogen *Cryptococcus neoformans* is the leading cause of fungal meningitis and is globally responsible for about 15% of AIDS-related deaths annually. An estimated 278,000 cases of cryptococcal meningitis occur a year, leading to roughly 181,000 deaths globally. This fungus can be found in many countries worldwide, predominantly in the soil. Typical individuals exposed to *C. neoformans* are capable of clearing inhaled spores, but immunocompromised people fall prey to prolonged lung infection where fungal cells can reside in immune cells and pass through the blood brain barrier where it causes lethal cryptococcal meningitis. The change from soil to lung offers a unique challenge for invading microbes, offering minimal glucose and alternative carbon sources such as acetate and lactate. Several metabolic changes occur to account for this, specifically upregulating genes related to acetate utilization and transport. Several acetate transporters across various fungi species belong to the ATO family. In *C. neoformans*, the three ATO family genes are *ATO1* (CNAG_05678), *ATO2* (CNAG_05266), and *ATO3* (CNAG_04787), which have yet to be fully characterized. In this study, we examine

phenotypes of single, double, and triple *ATO* mutants to understand their function(s) and potential role(s) in virulence.

Introduction

Invasive fungal infections cause nearly one and a half million deaths annually, accounting for nearly 50% of all AIDS-related deaths (Armstrong-James et al., 2014). Greater than 90% of all reported fungal deaths result from species of the genera *Cryptococcus*, *Candida*, *Aspergillus*, and *Pneumocystis*. The basidiomycetous fungus *Cryptococcus neoformans* is the most frequent cause of fungal meningitis (Brown et al., 2012) and is responsible for nearly half of the invasive fungal infections. *C. neoformans* plays a significant role in this risk by annually causing an estimated 278,000 cases of cryptococcal meningitis resulting in approximately 181,000 deaths globally according to the CDC (Rajasingham et al., 2017). These cases span across the globe, particularly in Asia and Sub-Saharan Africa and most often affect those that are immunocompromised (Rajasingham et al., 2017). Current treatments involve long treatment times, difficult delivery methods, and inefficient infection clearing (Perfect, 2010; Perfect, 2017). In fact, no effective antifungal treatments have been created in the last 30 years (Vanreppelen et al., 2023).

C. neoformans can survive under numerous environmental conditions which provides opportunities to infect and survive in new hosts that may offer these conditions (Lazera et al., 2000). This fungus is often found in the soil, certain species of trees, and in pigeon guano (Sorrell and Ellis, 1997; Emmons, 1955). Infection is established when spores or desiccated yeast are inhaled into the lungs (Chen et al., 2013). Individuals with healthy immune systems clear lung infections quickly, but immunocompromised individuals face many health challenges when infected. Individuals with apparently healthy

immune systems that struggle with a cryptococcal infection have been found to have a deficiency in antibodies that stimulate macrophages (Maziarz and Perfect, 2016; Chen et al., 2014). *C. neoformans* faces a new and dynamic environment in the lungs, and therefore begins a series of metabolic changes to survive in its new location (Hu et al., 2008). *C. neoformans* spreads beyond the lungs once it is phagocytosed by alveolar macrophages (Feldmesser et al., 2000). The phagolysosome is regulated by the ingested fungus to prevent its destruction which allows the crossing of the BBB using a "Trojan Horse" model, leading to devastating cryptococcal meningitis (Charlier et al., 2009).

During virulence, many metabolic pathways play an important role, especially glycolysis, β-oxidation, the citric acid cycle and glyoxylate shunt (Cherniak et al., 1998). Several genes related to glycolysis are upregulated in mouse lung tissues compared to in vitro growth conditions at 37 ºC such as phosphofructokinase, hexokinase, and enolase (Hu et al., 2008). Mice inoculated with pyruvate kinase (*pyk1*Δ) and hexose kinase I and II (*hxk1*Δ*/hxk2*Δ) mutants show higher survival (Price et al., 2011). Similarly, fatty acid β-oxidation produces energy and secondary metabolites that are of great importance (Hiltunen and Qin et al., 2000). Peroxisomal β-oxidation enzymes Mfe2 and Had1 have been shown to have a growth defect on fatty acids, reduced capsule growth, and restrained brain invasion when deleted in *C. neoformans* (Kretschmer et al., 2012). This leads to mice surviving twice as long in survival assays in comparison to the wild type (Kretschmer et al., 2012). Both β-oxidation and glycolysis need to be functional for virulence in *C. neoformans*.

Many fungi, including *C. neoformans*, are versatile in utilizing different nutrients and carbon sources which provide the ability to invade and survive in new environments and hosts. β-oxidation, the glyoxylate cycle, and other alternative pathways require carbon sources such as acetate or lactate to perform this task (Price et al., 2011). Acetate is imported and converted into acetyl-CoA by an acetyl-CoA synthetase (Acs) to be utilized in the citric acid cycle, the glyoxylate cycle, or in gluconeogenesis (Hynes et al., 2011). When lactate is imported into the cell, it is typically converted to pyruvate by a lactate dehydrogenase. This can then be transported into the mitochondria and enter the citric acid cycle (Guiard, 1985). This change in metabolism can provide advantages to fungal pathogens. *Candida albicans* upregulates several genes related to lactate and other alternative source metabolism when ingested by immune cells (Ene et al., 2012) and growth on alternative carbon sources such as amino acids or lactic acid confer resistance to important antifungals like caspofungin and fluconazole in other fungi (Williams and Lorenz, 2020).

When glucose as a carbon source is not available at early stages of infection, *C. neoformans* is thought to rely on acetate. SAGE data supports this possibility, with acetyl-CoA synthetase and other acetate metabolizing proteins upregulated in the absence of the sugar (Hu et al., 2008). Growth on acetate also alters gene expression in *Paracoccidioides lutzii* and *Paracoccidioides brasiliensis,* leading to upregulation of glyoxylate cycle genes. (Baeza et al., 2017). Proteomics studies in *Candida glabrata* show that enzymes involved in acetate metabolism, such as isocitrate lyase (Icl1), malate synthase (Mls1), aconitase (Aco1) and citrate synthase (Cit1), allow fungi to thrive within the human host (Chew et

al., 2021). *Aspergillus fumigatus*, another important pathogenic fungus, switches to using acetate when glucose is limited as seen by the regulation of transcription factors FacA and FacB, proteins important for acetate metabolism (Ries et al., 2021).

To utilize acetate, many fungi must rely on acetate transporters in glucose poor environments. In the yeast *Saccharomyces cerevisiae*, Ady2p is the acetate permease (Paiva et al, 2004). *Aspergillus nidulans* maintains production of AcpA, a protein that is important in transporting acetate and regulating pH (Sá-Pessoa et al., 2015). *C. albicans* maintains a large family of acetate transporters, some of which are transporting acetate out, rather than into the cell (Danhof and Lorenz et al., 2015). Many acetate transporters, whether transporting in or out, fall into a related family referred to as the ATO family. This family of genes was termed ATO or Ammonia Transport Out because of their release of ammonium found in *S. cerevisiae* (Řičicová et al., 2007).

C. neoformans has three ATO family proteins that share high identity (~50%) that are not fully understood. *ATO1* and *ATO2* show increased expression in murine lung tissue during early and onset infection (Hu et al., 2008). Lung tissue presents low levels of glucose while providing other carbon sources, such as acetate (Garnnett et al., 2012). An *ato1* mutant grown on acetate as the sole carbon source shows a growth defect (Grace Kisirkoi and Kerry Smith, unpublished). In invertebrate survival assays, there is a strong trend of virulence attenuation in *ato1* and *ato1ato2* mutants compared to wild type (Grace Kisirkoi and Kerry Smith, unpublished). In mice, only *ato1ato2* mutants were attenuated compared to the wildtype and single mutants (Charles Specht, Grace Kisirkoi, Kerry Smith, and

Stuart Levitz, unpublished). The *ato1ato2* mutant exhibited several other phenotypes that the corresponding single mutants did not display, such as lack of thermotolerance, melanin production, and capsule production. This led to doubts about the validity of the Kronstad mutants. In this study, we aim to confirm these phenotypes to ensure that they are not caused by unmarked off-target edits by generating new double and triple deletion mutations of the *ATO1*, *ATO2*, and *ATO3* genes starting from a different single mutant library. Here, we have examined common virulence factors and found that *ato* mutants show variations in melanin production, sensitivity to SDS exposure, a smaller capsule size, changes in growth on pH adjusted media, and changes in survival in the invertebrate model of infection *Galleria mellonella*.

Materials & Methods

ATO Deletion Mutant Generation

Double and triple mutants were created using Transient CRISPR-Cas9 coupled with Electroporation (TRACE) (Lin et al., 2020) starting with strains from the Madhani knockout library (Chun and Madhani, 2010). Donor DNA containing 50 bp flanks with the gene of interest and 20 bp of an antibiotic cassette was amplified from pKS1 using PCR. A transient Cas9 cassette and target gRNA were amplified from a plasmid, either pKS1 or pSDMA57. Cells grown overnight were transformed via electroporation and recovered on selection plates. Colonies were tested for genomic stability over five days as previously described (**Supplemental Figure 2.1**) (Lin et al., 2020). The workflow of which gene was knocked out in which starting strain is indicated in **Supplemental Figure 2.2**. RNA was extracted via BeadBeater using a RiboPure Yeast kit (Invitrogen, USA) following the kit instructions. Deletions were qualified using QIAGEN One-step RT-PCR Kit.

Stress Plate Assays

Plates for testing various stressors were prepared by making and autoclaving YNB with amino acids (YNB-AA) base media and adding the stressor after allowing the media to cool. The following stressors were used: 0.5% Congo Red, 1.5 mg/mL Calcofluor White, 0.06% SDS, 1.5 M NaCl, or 1.2 M KCl. Cells were grown and plated as described in the spot assay protocol.

pH indicator plates were made by adding components (10% carbon source, 10 X YNB-AA, ddH_2O) and then adjusting the pH with sodium hydroxide or hydrochloric acid. Agar and a stir bar were added before autoclaving. After autoclaving and cooling, 0.01% bromocresol purple was added and plates were poured. Cells were grown and plated as described in the spot assay protocol. Images were taken at 24, 48, and 72 hours using a Canon EOS Rebel T1i. Reagents and materials were purchased from Fisher Scientific, VWR Scientific, Sigma Aldrich, and Gold Biotechnology.

Melanin Production Assay

Melanin plates were prepared by making YNB-AA base media and adding the melanin induction media after cooling. Final melanin media concentrations are as follows: 8 mg/mL KH_2PO_4, 2 mg/mL glucose, 2 mg/mL L-glycine, 1 µg/mL D-biotin, 1 µg/mL thiamine, 0.92 mg/mL $MgSO_4$ 7H2O, and 0.4 mg/mL L-DOPA. Cells were grown and plated as described in the spot assay protocol. Images were taken at 24, 48, and 72 hours using a Canon EOS Rebel T1i.

Spot Dilution Assay Protocol

Samples from freezer stocks were grown in YPD and shaken at 30ºC overnight. Cells were refreshed the next day in fresh YPD, and the Abs_{600} was checked to ensure Abs_{600} less than 0.1. Cells were incubated for three hours to an Abs_{600} between 1.0 and 2.0. Cells were collected and washed in Dulbecco's phosphate buffered solution (DPBS). Three dilutions (1:10, 1:100, and 1:1000) were made using DPBS. Cells were vortexed and 2 µL spots from each dilution and the original tube were plated. Plates were incubated at both 30ºC and 37ºC. Images were taken at 24, 48, and 72 hours using a Canon EOS Rebel T1i.

Growth at different pH values

YNB with a 2% carbon source was adjusted to a pH of 4, 6, or 8 using sodium hydroxide or hydrochloric acid. A YPD culture was prepared from freezer stocks and allowed to grow overnight. The cells were collected and washed in DPBS the next day. Cells were counted via hemocytometer, adjusted to 150 cells/µL, spun down, and vortexed in 1 mL of pH adjusted media. Cells were placed in a 96 well plate with shaking at 250 rpm at 30ºC. Readings were taken every 20 minutes for 72 hours with the BioTek LogPhase 600. Data points were plotted using Prism (GraphPad). A 1 way ANOVA was used to compare curves where $\alpha = 0.05$. Individual curves were compared using Tukey's multiple comparison test where $\alpha = 0.05$.

RMPI CO_2 Assay

RPMI 1640 was buffered with 165 mg/mL MOPS. YPD liquid cultures were made from freezer stocks and allowed to grow overnight. The cells were collected, washed in

DPBS, and equalized via absorbance at 600 nm. Cells were vortexed in 1 mL of RPMI and placed in 96 well plates. Cells were shaken at 250 rpm at 37ºC in either ambient conditions or 5% CO_2. Optical density readings were taken every 24 hours for 72 hours using a BioTek Epoch microplate reader. Data was graphed using Prism (GraphPad). A 1 way ANOVA was used to compare curves where $\alpha = 0.05$.

Capsule Induction Assay

YPD overnight cultures were made from freezer stocks of each mutant strain and WT. The next day, 100 μL of each culture was collected, centrifuged, and washed with DPBS. Cells were resuspended in 10% FBS in Dulbecco's Modified Eagle Medium (DMEM) and placed in a non-treated 48 well plate. Plates were incubated for two to three days in 5% CO_2 at 37 C. After incubation, cells were collected, and washed and resuspended in DPBS. India ink, then cells were added to a slide and covered. Cells were imaged using a 100x oil immersion objective on a Leica DMi8. The body and capsule diameter of cells were measured using LASX Office software and graphed in Minitab. Cell radii were compared using a paired T-test where $\alpha = 0.05$.

$$\text{Capsule radius} = (\text{total cell diameter} - \text{cell body diameter}) / 2$$

Galleria Survival Assay

Cells from freezer stocks were made into overnight YPD liquid cultures. Cells were collected, washed in DPBS, and resuspended in DPBS. From here, an aliquot was kept in a microcentrifuge tube for injection. A separate sample was placed in a non-treated 96 well plate to be counted in a CytoFLEX flow cytometer (Beckman). Each tube was then equalized to 10,000 cells/µL. A portion of the WT sample was heat killed by placing it at 65ºC for one hour. Spotless, milky-white *Galleria mellonella* larvae weighing between 0.25 g and 0.30 g were selected. *G. mellonella* left prolegs were sanitized via ethanol and injected with 10 µL cells using a sterilized Hamilton syringe. Syringes were washed in bleach, ethanol, DPBS, and sterile water between injections. 10 larvae were injected per strain. The injected larvae were incubated at 37ºC and checked every 24 hours for 10 days. Surviving larvae were counted at each observation. Results were displayed using Prism (GraphPad) to create Kaplan-Meier survival plots. A log-rank (Mantel-Cox) test was used to compare survival curves.

Results

Mutant generation

Previously observed inconsistent phenotypes of Kronstad lab single and double mutants, such as low thermotolerance, lack of capsule formation, and contrasting survival results in *Galleria mellonella* and mice led to concerns about the validity of the strains.

Perhaps these strains contained undiscovered off-target edits or mutations. We would expect that a double mutant exhibits a phenotype similar to or summative of its constitutive single mutants. Because of this, we regenerated double mutants and a triple mutant starting with single mutants from the Madhani library using CRISPR-Cas9 and qualified the knockouts via RT-PCR (**Supplemental Figure 2.3**). The validity of the *Δato1ato3* strain was brought into question when a band for the actin positive control was not visible. This suggests that the RNA sample, and/or the strain itself, may be compromised.

Carbon source growth

We utilized several carbon sources to survey *ato* mutant growth as a means to investigate the role of Ato1, Ato2, and Ato3. Gene information is shown in **Table 2.1**. Previous work in our lab has shown that *Δato1* struggled to grow on acetate as a sole carbon source compared to the wild type, especially at lower concentrations such as 0.5% and 0.2% (Grace Kisirkoi and Kerry Smith, Unpublished data). This was also seen in the *S. cerevisiae* acetate permease Ady2p mutant (Paiva et al., 2004). Here we tested acetate, lactate, pyruvate, succinate, and propionate as sole carbon sources in a YNB-AA base media at 30°C and 37°C. We found that each Madhani single mutant grew similar to wild type on all carbon sources tested except for acetate on plates (**Table 2.2**). On acetate, we saw that the *Δato1* strain grew less well on acetate at both 30°C and 37°C (**Figure 2.1**). These results support the idea that Ato1 is an acetate transporter. Ato1 analogs in other fungi function similarly and previous work in our own lab examining uptake of

radiolabeled acetate is consistent with this. (Grace Kisirkoi and Kerry Smith, Unpublished data).

Δato3 mutants are more sensitive to plasma membrane stressor SDS than wild type

In addition to various carbon sources, we also looked at several cell wall, plasma membrane, and osmotic stressors. These experiments were carried out to mimic different stresses that a fungal pathogen encounters during host invasion. We found that all strains grew normally compared to the wild type on 1.5 M NaCl and 1.2 M KCl (not shown), except for *Δato2ato3* on NaCl at 37°C (**Figure 2.2A**). All strains also grew normally compared to the wild type on 0.5% Congo red and 1.5 mg/mL Calcofluor White (CFW) at 30°C and 37°C (not shown). Strains missing *ATO3*, except for *Δato1ato3*, were much more resistant to SDS than other mutants and the wild type (**Figure 2.2B**).

Ato mutants show variable growth on pH plates and liquid media

ATO genes in *C. albicans* have been shown to play an important role in alkalizing the environment by exporting ammonia (Carman, 2008; Danhof and Lorenz et al., 2015). Ato proteins could also play an important role in alkalizing or acidifying the environment. We recreated experiments used to observe growth in pH adjusted media and environmental pH regulation to identify *ato* mutants that struggled to alkalize the environment. Ato mutants showed an additive growth advantage over the wild type on 2% glucose YNB-AA (pH 4, 6, 8) We saw no changes in growth on 2% glucose YNB-AA plates adjusted to pH 4 or 6 (not shown). Mutant strains and the wild type grown on 2% acetate YNB-AA (pH

2, 4, 6) 30°C show no growth (**Figure 2.3**). We did see that *ato* single mutants grow better than the wild type on 2% glucose YNB-AA (pH 8) plates and liquid media at 30ºC (**Figure 2.4A**). We also saw that *Δato1* struggled to grow on 2% acetate YNB-AA (pH 8) compared to the wild type and other single mutants (**Figure 2.4B**). Strains missing *ATO1* showed little to no growth defect on pH 6 on 1% acetate as sole carbon source (**Figure 2.5**). *Δato3, Δato1ato3, and Δato2ato3* mutants grew slightly better than other strains and the wild type on 1% acetate YNB-AA (pH 6) 37°C (**Figure 2.5**). Strains missing *ATO1* showed a growth defect on pH 8 on 1% acetate as sole carbon source at 30ºC and 37ºC (**Figure 2.6**). Strains grown on 1% acetate YNB-AA (pH 4) do not grow at 30°C or 37°C (not shown).

Ato mutants produce visible melanin sooner than the wild type strain

Melanin production is another critical virulence factor that protects *C. neoformans* against oxidative stress and phagocytosis (Zhu and Williamson, 2004). We found that all *ato* mutant strains produced visible melanin sooner than the wild type at 30°C (**Figure 2.7**). This result was not seen at 37°C, rather each mutant produced melanin at the same rate as the wildtype on 0.06% SDS at 37°C.

***ato* mutants show reduced capsule production, with double mutants showing an additive effect**

A polysaccharide capsule is produced early in lung infection by *C. neoformans* and is used for protection from immune cells (Bojarczuk et al., 2016; Casadevall et al., 2019; Coelho et al., 2014). Mutant strains that struggle to produce a capsule are also avirulent

(Vecchiarelli et al., 2013). We measured capsule production in *ato* mutant strains (N = 50) using light microscopy and LASX Office software (Leica). We found a statistically significant difference between the wild type strain and each *ato* mutant strain ($p \leq 0.0001$ using $\alpha = 0.05$ using paired T-test) (**Figure 2.8A**). We also discovered that each double mutant capsule radius is significantly decreased compared to the corresponding single mutants. We found a similar effect when examining the triple mutant compared to the wild type strain (**Figure 2.8B**).

ato mutants grow normally in minimal conditions that mimic the *in vivo* environment

It has been shown that *C. neoformans* mutants that have difficulty growing in RMPI 1640 minimal media incubated at 37°C in 5% CO_2 correlate with higher virulence (Ristow et al., 2023). We utilized RPMI media buffered with MOPS to investigate if *ATO* genes were necessary for survival and growth in 5% CO_2 in RPMI media. We found that *ato* mutants grew normally under these conditions compared to the wild type strain (**Figure 2.9A**). We did not find any relationship between the growth of *ato* mutants in ambient conditions and growth in CO_2 (**Figure 2.9B**)

Mutants missing *ATO2* and *ATO3* show strongest survival in an invertebrate model

The greater wax moth (*Galleria mellonella*) larval stage has proven to be a useful model of bacterial and fungal infection (Mylonakis et al., 2005). We injected *G. mellonella* with each *ato* mutant strain and incubated them at 37°C to mimic the mammalian host temperature. We found that *G. mellonella* injected with *ato* single mutants fared worse than

the wild type strain or the *Δato1ato2* mutant (**Figure 2.10**). The *Δato1ato3* mutant injected invertebrates survived slightly better than the wild type, with the *Δato2ato3* and *Δato1ato2ato3* infected larvae displaying the longest survival compared to the wild type and other mutant strains. The larvae injected with DPBS died quicker than the untouched larvae, leading us to believe that the DPBS exhibited osmotic stress on the larvae. Other researchers likely dilute DPBS before injection to prevent this. The larvae injected with heat-killed cells also died very quickly, likely indicating that the cells were not completely heat-killed. The single mutants and double mutants, except for *Δato1ato3* and *Δato2ato3*, resulted in death faster than the wild type.

Discussion

C. neoformans can thrive in various environments due to its metabolic flexibility and regulation of its surroundings. This allows for greater virulence and makes identifying effective treatments difficult (Lazera et al., 2000; Perfect, 2010). *C. neoformans* faces a new and challenging environment in the lungs that it must contend with by utilizing acetate and other alternative carbon sources to glucose. Virulence requires many metabolic pathways especially glycolysis, gluconeogenesis, β-oxidation, the citric acid cycle and glyoxylate shunt (Cherniak et al., 1998). It is known that several genes related to acetate utilization are upregulated *in vivo*, including putative acetate transporters *ADY2* and *ATO2*

(Hu et al., 2008). Acetate transporters have been shown to be necessary when grown on low concentrations of acetate and higher pH in many organisms (Wolfe, 2005) and likely play a critical role in surviving within a host. As it turns out, many acetate transporters and genes thought to encode for acetate transporters belong to the ATO family of proteins and are involved in pH regulation and signaling. In this study, we highlight common virulence factors, stress resistance, and environmental pH regulation of the three ATO genes found in *C. neoformans* along with double and triple mutants of these genes.

Previous work in our lab found that *Δato1* mutant strains did not grow well on acetate, and that Ato1 was required for uptake of radiolabeled acetate. This work also found that an *Δato1ato2* mutant strain struggled to grow in 37ºC and to form a capsule. Survival was not consistent in Galleria and mice. Larvae inject with *Δato1* survived quite well, but mice did not. Also of note, when Ato1 and Ato2 are missing almost all Galleria and all mice survived survival assays, indicating that these proteins appear to be critical for virulence (Charles Specht, Grace Kisirkoi, Kerry Smith, and Stuart Levitz, unpublished).

Initially, we were able to confirm that *Δato1* did not grow on acetate, especially lower concentrations of acetate (**Figure 2.1**), as seen among Ato1 analogs in other fungi, but we did not find that Ato2 or Ato3 were directly transporting acetate under the experimental conditions. This led us to screen several other short chain carboxylic acids such as lactate and propionate in which we did not see any growth defects (**Table 2.3**). These results led us to conclude that the Ato transporters, especially Ato2 and Ato3, were not directly involved in transporting these alternative carbon sources. We did notice that all *ato* single mutants were able to grow at 37 C along with the wild type, indicating that

the genes are not individually responsible for thermotolerance, a critical virulence factor. In the future, carbon source screening could be carried out again at lower concentrations to verify that Ato2 and Ato3 are not important for low concentration transport of the molecules examined aside from acetate, which we have already investigated.

As previously mentioned, capsule production is a critical virulence in *C. neoformans*. Strains that have limited ability to produce a capsule are avirulent (Vecchiarelli et al., 2013) due to the importance of the capsule in protecting the fungus from immune cells (Casadevall et al., 2019). We see that there is a statistically significant difference in capsule growth between the wild type, single, and double mutants (**Figure 2.8A**). We also see that there is a significant difference in capsule size between double mutants and their constitutive single mutants. This indicates to us that there may be an additive defect where each Ato plays a role in capsule formation or integrity. The more Atos are missing, the poorer the capsule production is. The triple mutant capsule formation was also found to be smaller than the wild type (**Figure 2.8B**) which continues to indicate that these proteins may play a role in capsule formation. These experiments should be repeated with all mutant strains in one experiment so comparisons can be made between single, double, and triple mutants.

Sodium dodecyl sulfate (SDS) is used as a plasma membrane stressor to imitate stresses similar to those found in a human host (Elorza et al., 1983). While we examined various stress environments, we only saw major changes in growth on SDS. Mutants missing *ato3* showed enhanced growth on 0.06% SDS, except for *Δato1ato3*. Ato3 seems to be important for the structural integrity of the plasma membrane in capsule formation

and also as a target of detergent mediated plasma membrane stress. Ato transporters may generally play a role in plasma membrane integrity. In other results, we see that these proteins seem to inhibit common virulence factors which may explain why *Δato1ato3* does not show the growth phenotype on SDS like other *ato3* mutants.

Recent studies have shown that growth in 5% CO_2 could be another relevant virulence factor because high virulence clinical strains grow well in these conditions, but low virulence strains grow poorly in 5% CO_2 (Krysan et al., 2019). We examined *ato* mutants under these conditions using buffered RPMI. We found that *ato* mutants grew normally compared to the wildtype in RPMI at both ambient conditions and at 5% CO_2 (**Figure 2.9**). This likely means these proteins are not involved in regulating growth in relation to high CO_2 conditions that are found during infection.

Melanin protects the cell against oxidative stress and phagocytosis which marks its production as a valuable virulence factor (Zhu and Williamson, 2004). We found that *ato* mutants produce melanin at a faster rate on plates at 30°C than the wildtype (**Figure 2.7**). We did not see this at 37°C. Ato proteins appear to delay melanin production at 30°C but not at 37°C which may indicate that there is a temperature sensitive response to their absence.

In other fungi, there is a precedent that ATO family proteins play a role pH regulation and signaling (Palková et al., 2002; Sá-Pessoa et al., 2015; Danhof and Lorenz et al., 2015). Because of this, we examined growth on pH adjusted media. We found that *ato* single mutants grow better than the wildtype on 2% glucose YNB-AA (pH 8) plates and liquid media at 30ºC. We also saw variance in growth on 2% and 1% acetate YNB-

AA plates. *Δato3, Δato1ato2,* and *Δato1ato3* mutants grew slightly better than other strains on 1% acetate YNB-AA (pH 6) at 37°C. Strains missing *ato1* showed a growth defect on 1% acetate YNB-AA (pH 8) which aligns with other acetate experiments finding that strains missing *ato1* had a growth defect on various concentrations of acetate. The variation of growth on acetate can likely be attributed to the form of acetate depending on the pH of the media. The *Δato3, Δato1ato2,* and *Δato1ato3* results raises more questions on the roles of these trasnporters in growth on acetate. Perhaps these strains allow for a higher acetate metabolism, but these experiments need to be optimized to gain clearer insights.

Galleria mellonella offers an inexpensive means of modeling fungal infection (Mylonakis et al., 2005). When injected with *ato* single mutants, *G. mellonella* show impeded survival compared to the wild type. We see that in some cases, *ATO* genes individually impede virulence rather than promote it. This result may link with the results from our melanin experiments in which missing Ato transporters enhance the rate at which melanin is produced at 30°C compared to the wildtype. However, we did not see this result at 37°C. The larvae injected with *Δato2ato3* and *Δato1ato2ato3* mutants survived the longest, leading us to believe that there is a relationship between Ato2 and Ato3 which is important for virulence. This result is notable in concert with the results from our SDS experiments showing that Ato3 appears to be important as a target for detergent stress. These experiments do need to be repeated in a more robust manner where both 30°C and 37°C are examined, and the fungal load is reduced to a biologically relevant level.

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Tables

Table 2.1: List of *ATO* genes

Organism	Transporter name	Identification number	Chromosome	Predicted Location
C. neoformans	Ato1/Ady2	CNAG_05678	7	Plasma Membrane
	Ato2	CNAG_05266	4	Plasma Membrane
	Ato3	CNAG_04787	10	Plasma Membrane

Table 2.2: Primers for Creation of Mutants, PCR, and RT-PCR

Primer	Sequence	Use
Ato2 HYG-F	TTCGTCAGATTGCAGCAATATTGCGATTAC TTGGTATCGGTTATTTGGGC	Amplification of donor DNA
Ato2 HYG-R	GAGTGGGTGTGTCACGAGCCTGCACGAAGT AGAAGAAATAAGAGATCATA	Amplification of donor DNA
Ato3 NEO-F	GTGCCTGAATATGATGTGTGC GACGTCCGTGTGATTTACACCATCGTCAT AATCGCCTTGCAGCACATCC	Amplification of donor DNA
Ato3 NEO-R	GGCATTGTCACCATGGTACACC AAGGACAGTCAAGACAAATGGTTAATTCC TTGAGCGTGCTTCATTGGCC	Amplification of donor DNA
gRNA-F	CCATCGATTTGCATTAGAACTAAAAACAAAGCA	Amplification of gRNA construct
gRNA-R	CCGCTCGAGTAAAACAAAAAAGCACCGAC	
Cas9-F	GGTGACGCTGTGAGAGTGG	Amplification of Cas9 construct
Cas9-R	GGGCCCCTCTTCACGTGG	
Ato2 RT-F	GCAAATCAGGCTATAATCTATG	RT-PCR for *Ato2* (CNAG_05266)
Ato2 RT-R	AAAAGGTCTACACTCAATAC	
Ato3 RT-F	TGACAACTTCAGCTAATCACTCG	RT-PCR for *Ato3* (CNAG_04787)
Ato3 RT-R	CGTAGGCACAAGGAAGAAAC	
Actin RT-F	CTATCCTCCGTATCGATCTT	RT-PCR control
Actin RT-R	TCTGCTGGAAGGTAGACAAA	

Table 2.3 Single *ato* Mutant Growth on Carbon Source Plates

	Δato1	*Δato2*	*Δato3*
Acetate	**Defect**	**No Defect**	**No Defect**
Lactate	**No Defect**	**No defect**	**No Defect**
Pyruvate	**No Defect**	**No Defect**	**No Defect**
Succinate	**No Defect**	**No Defect**	**No Defect**
Propionate	**No Defect**	**No Defect**	**No Defect**

Figures

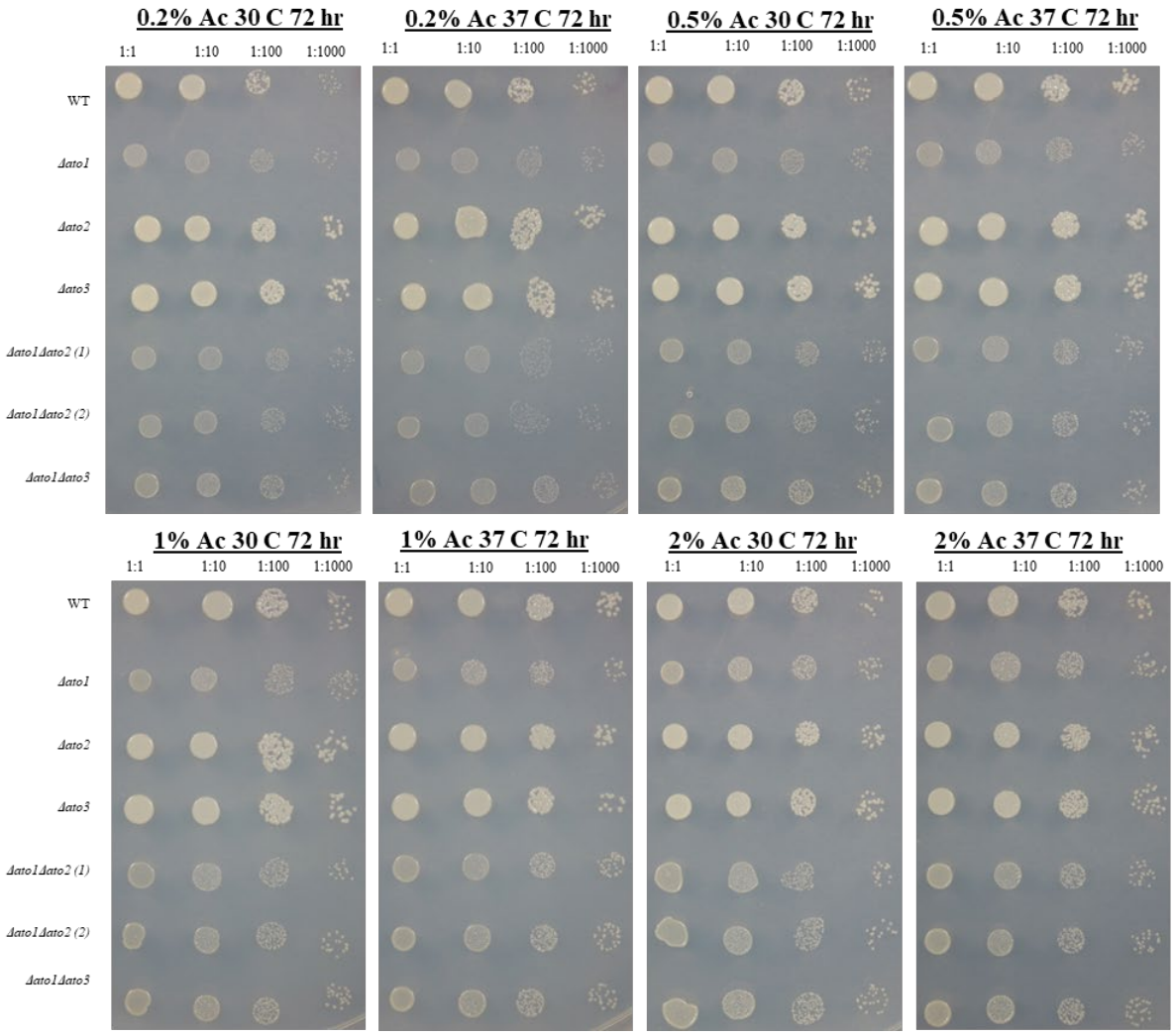


Figure 2.1: Mutants missing *ATO1* show a growth defect when acetate is the sole carbon source. Fungal strains were grown on YNB+AA agar with the indicated percentage (w/v) of acetate. Numbers at the top indicate culture dilution. Strains were incubated at 30 ℃ or 37 ℃ for 72 h and imaged. Two strains of *Δato1ato2* are shown here.

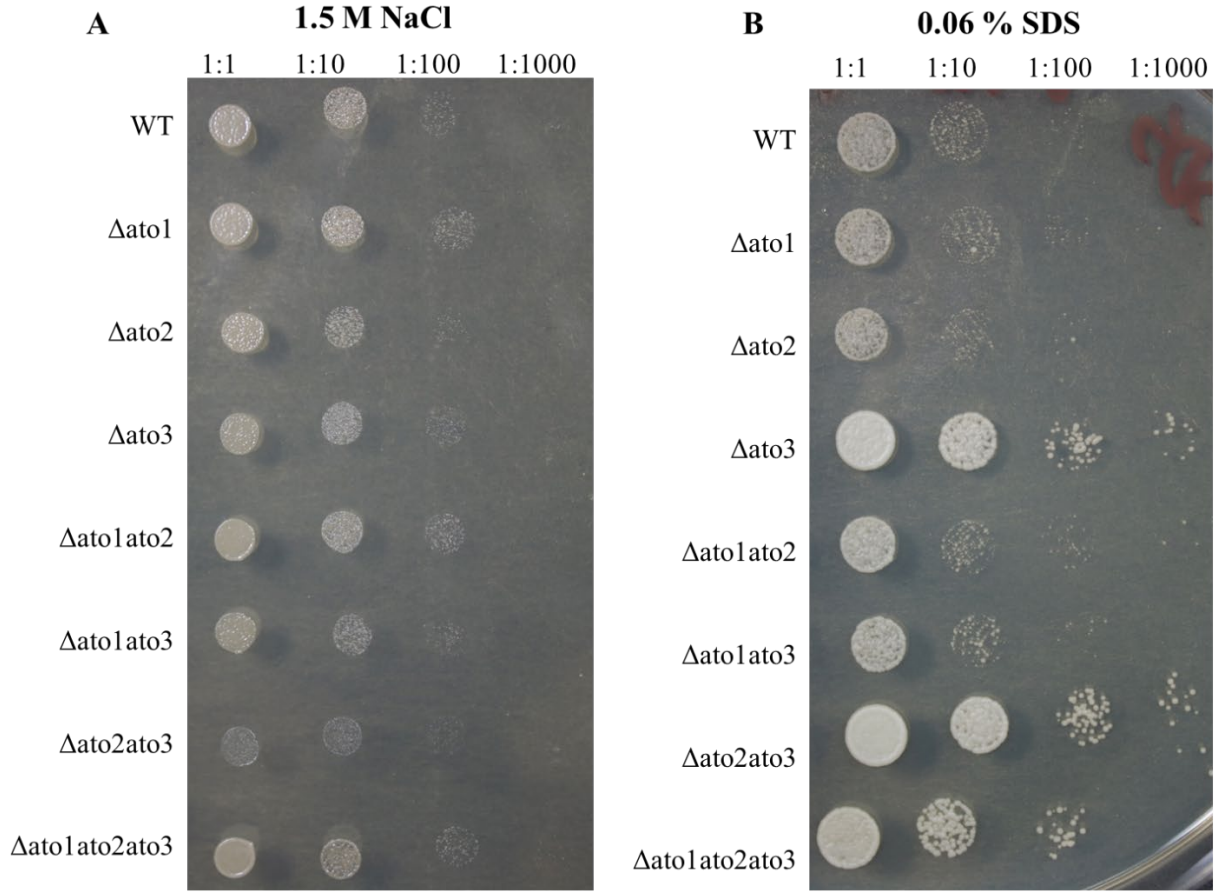


Figure 2.2: A. Most *ato* mutants are not sensitive to osmotic stress. B. Ato3 mutants are sensitive to SDS. Fungal strains were grown on YNB+AA agar with the indicated stressor. Numbers at the top indicate culture dilution. Strains were incubated at 37°C for 72 h and imaged.

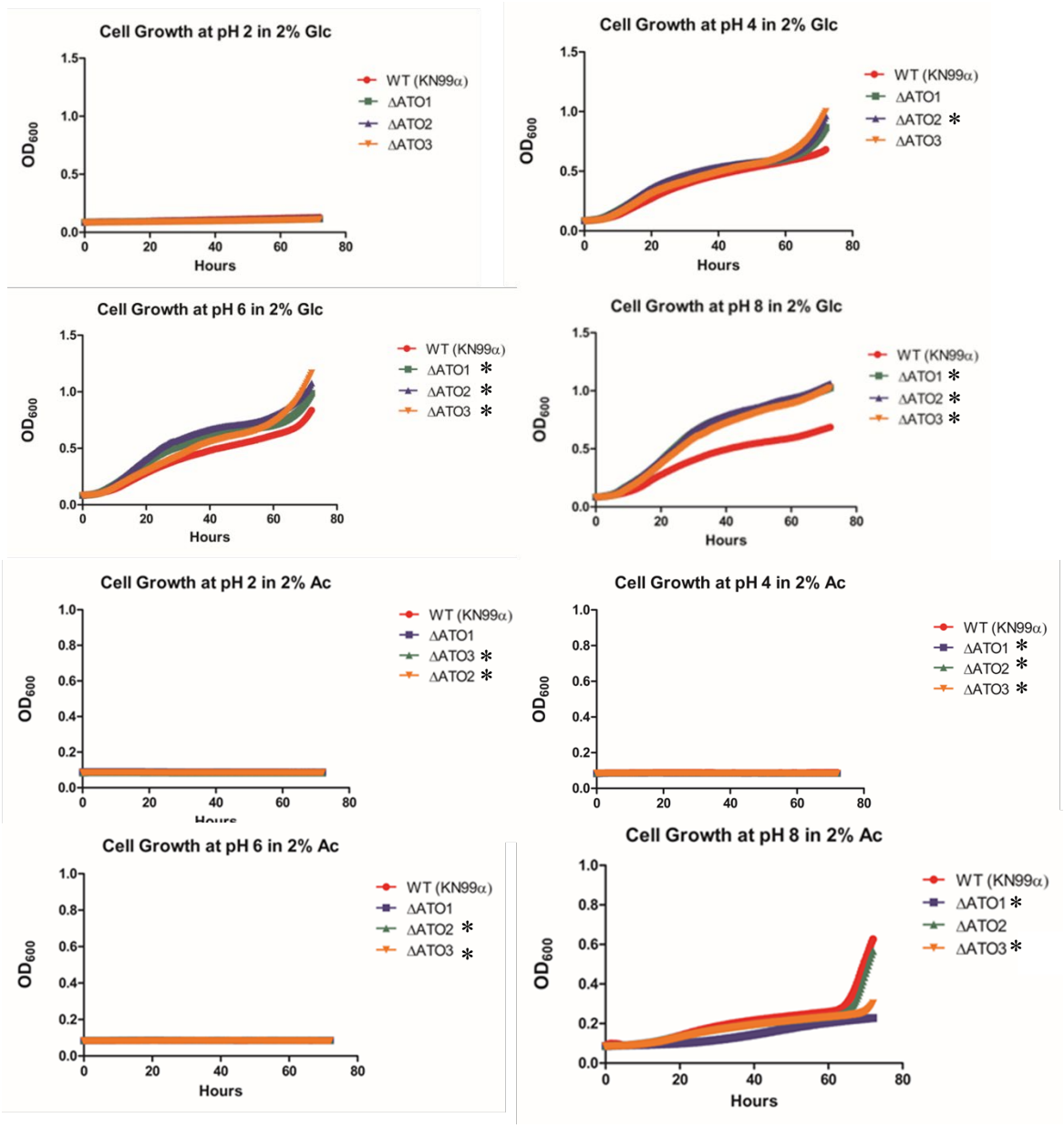


Figure 2.3: *ato* single mutants show enhanced growth on glucose compared to the wildtype in pH adjusted media. Liquid YNB-AA with 2% (w/v) glucose or acetate was adjusted to the pH of 2, 4, 6, or 8. Fungal strains and media were placed in a 96 well plate and read using a Log600 plate reader every 20 minutes for 72 hours. Plates were shaken at 250 rpm in 30ºC. Each trial was found to be significantly different via 1 way ANOVA

where $\alpha = 0.05$ and $p \leq 0.0001$. * indicates significant difference from the wild type using Tukey's multiple comparison test where $\alpha = 0.05$.

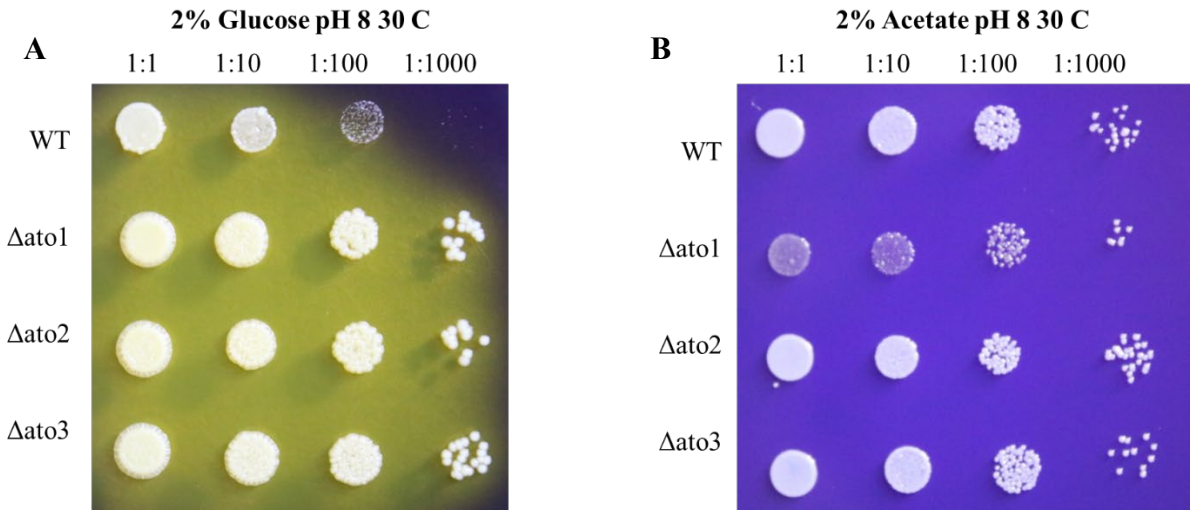


Figure 2.4: *ato* single mutants show more growth on pH 8 glucose. Indicator plates were made using YNB-AA media with carbon source, then adjusting the pH and adding bromocresol purple as an indicator. Numbers at the top indicate culture dilution. Strains were incubated at 30 °C for 72 hours and imaged.

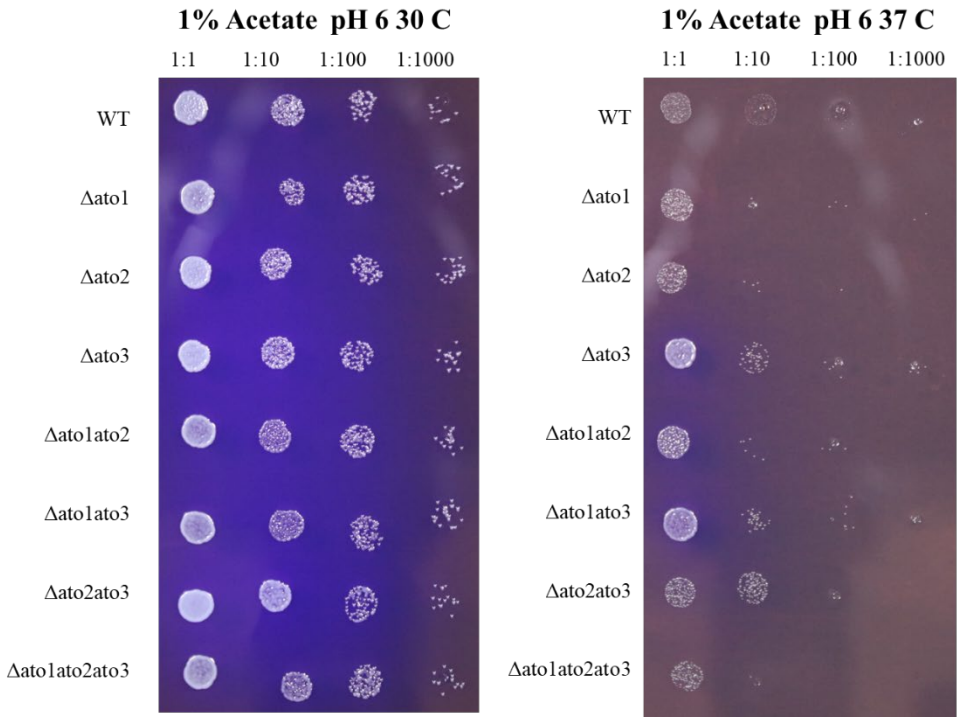


Figure 2.5: Mutant strains grown on pH 6 1% acetate 30 C show no growth defect. *ato* mutants show more growth on pH 6 1% acetate. Indicator plates were made using YNB-AA media with carbon source, then adjusting the pH and adding bromocresol purple as an indicator. Numbers at the top indicate culture dilution. Strains were incubated at 30 ºC or 37 ºC for 72 hours and imaged.

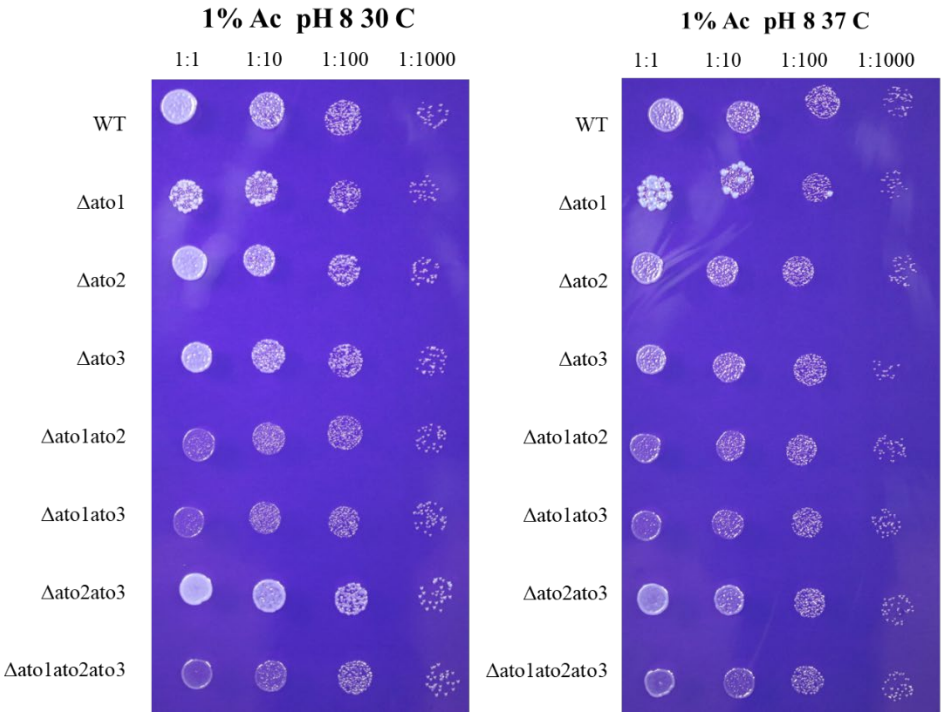


Figure 2.6: Strains missing *ATO1* showed a small growth defect on pH 8 on 1% acetate. pH indicator plates were made as previously described above. Strains were grown at 30ºC or 37ºC and imaged at 72 hours.

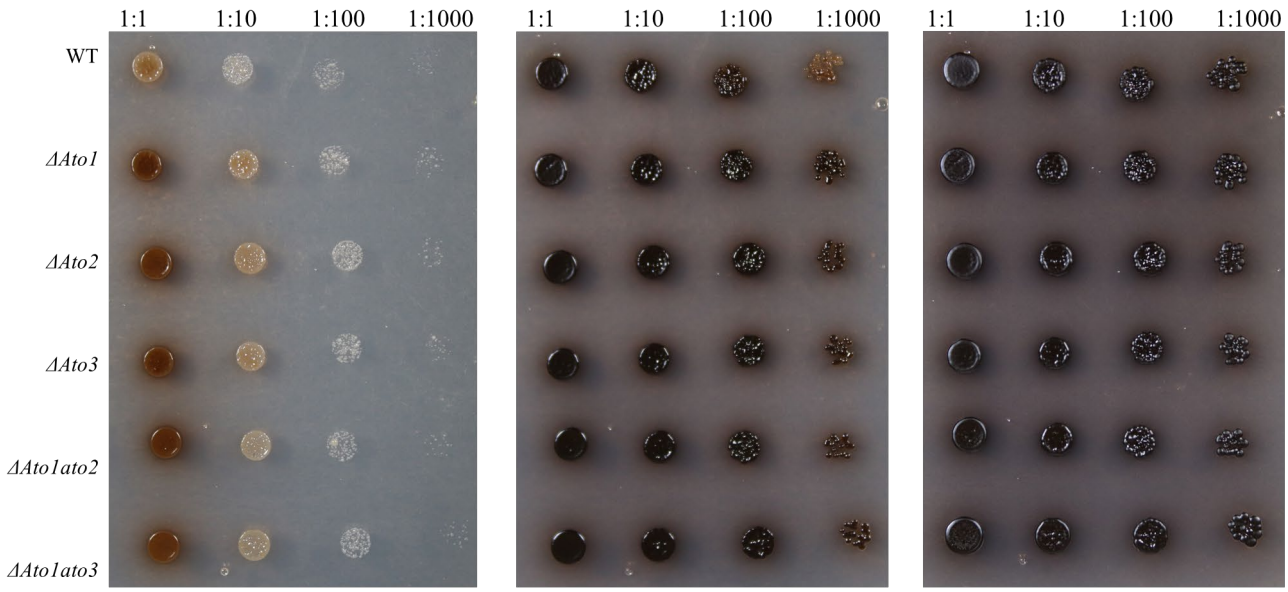
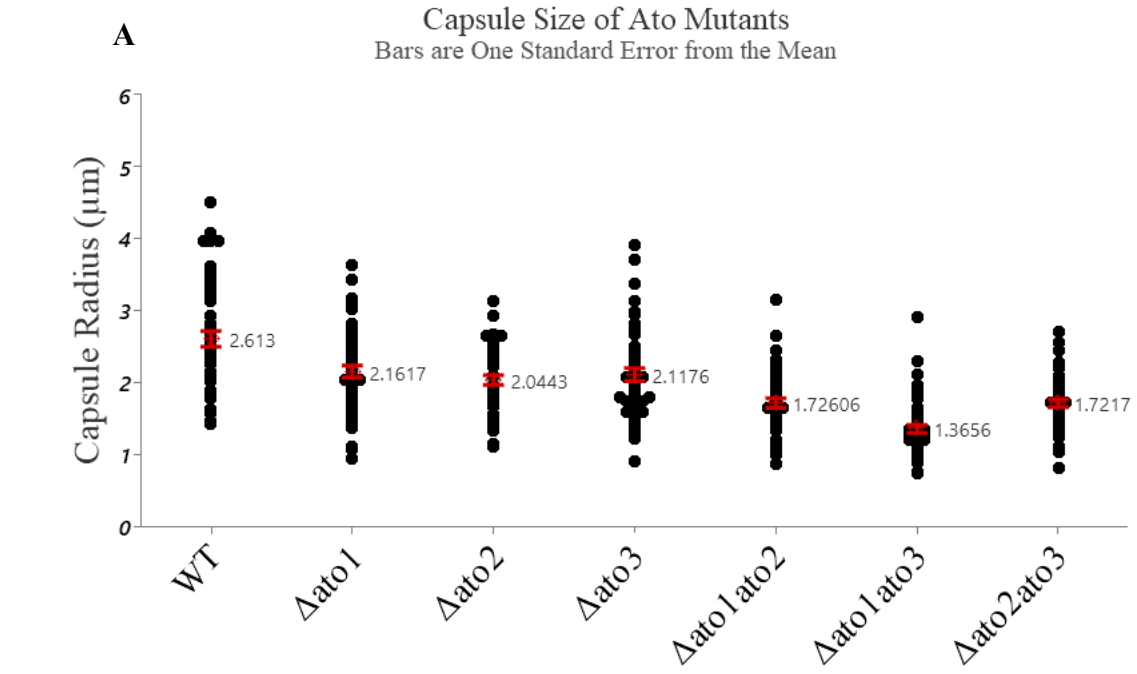


Figure 2.7: *ato* mutants produce visible melanin sooner than the wild type strain.

Melanin production was observed at 30˚C on L-3.4 dihydroxyphenylalanine (L-DOPA) agar induction plates. Numbers at the top indicate culture dilution. Strains were incubated and imaged at 24, 48, and 72 hours. 2 mg/mL glucose is included in the melanin induction media.


A
Capsule Size of Ato Mutants
Bars are One Standard Error from the Mean
Capsule Radius (µm)
0
1
2
3
4
5
6
2.613
2.1617
2.0443
2.1176
1.72606
1.3656
1.7217
WT
Δato1
Δato2
Δato3
Δato1ato2
Δato1ato3
Δato2ato3

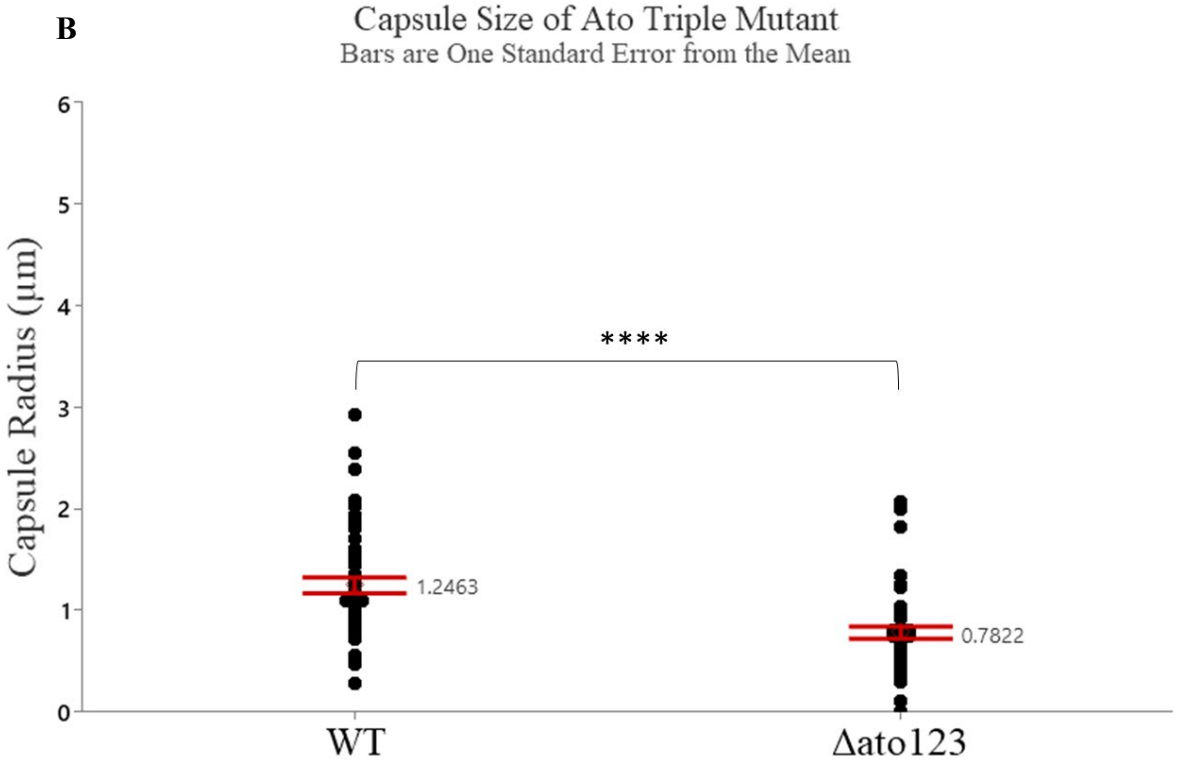

B
Capsule Size of Ato Triple Mutant
Bars are One Standard Error from the Mean
Capsule Radius (µm)
0
1
2
3
4
5
6
****
1.2463
0.7822
WT
Δato123

Figure 2.8: A. *ato* mutants show reduced capsule production, with double mutants showing an additive effect. Cells were grown in capsule induction media (DMEM + 10% FBS) at 37ºC in 5% CO_2 for 2-3 days. Cells were negatively stained in india ink and measured using a Lieca DMi8 and LAS X Office software. Each mutant showed a statistically significant difference in capsule size compared to the WT. This is also true of each double mutant compared to their constitutive single mutants. $N = 50$. $p \leq 0.0001$ using $\alpha = 0.05$ using paired T-test. **B. *ato* triple mutant shows significantly smaller capsule size compared to the wildtype.** Capsule production was induced and measured as previously mentioned. $N = 50$. $p \leq 0.0001$ using $\alpha = 0.05$.

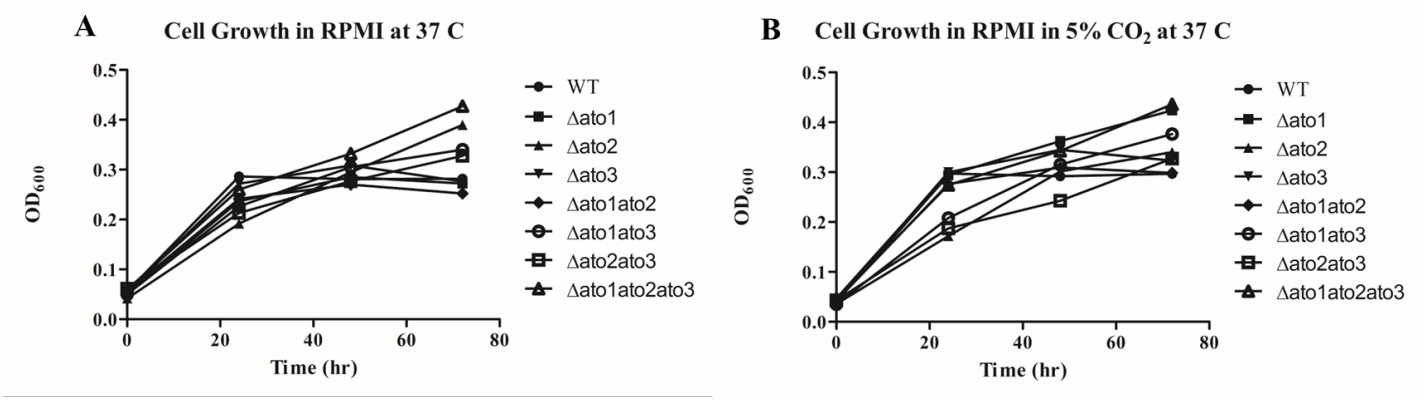
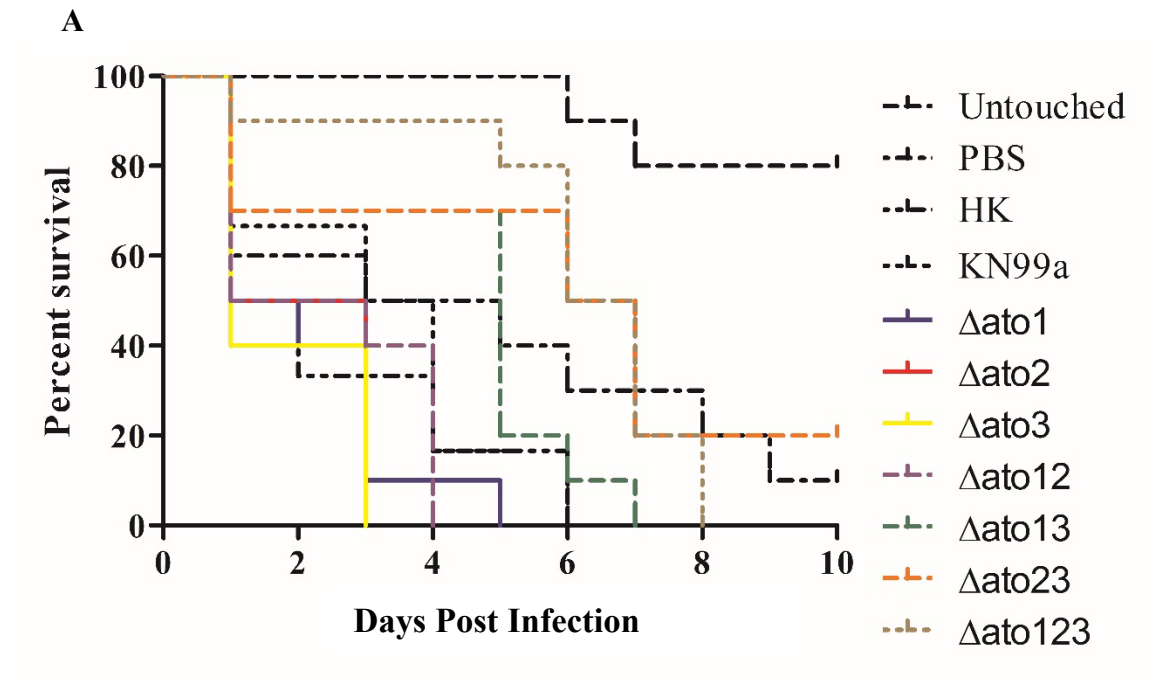
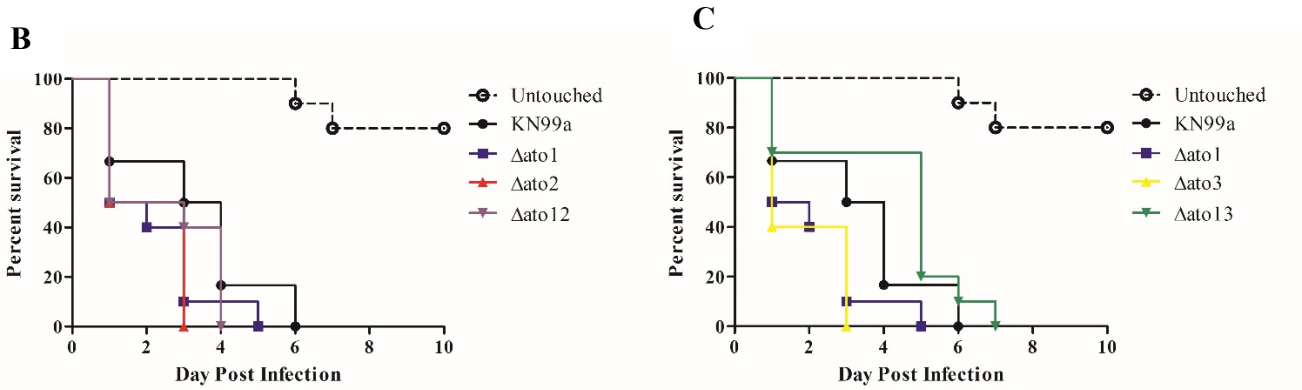
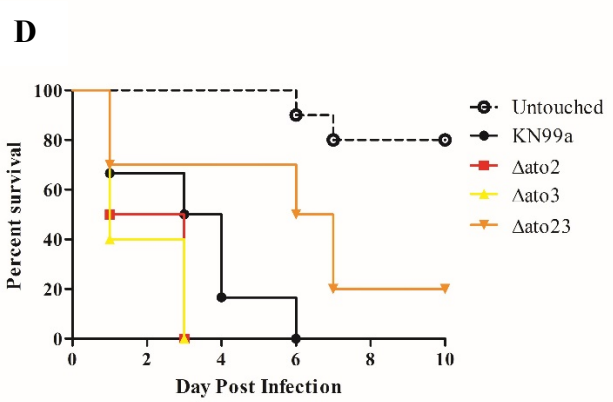


Figure 2.9: *ato* mutants grow normally in 5% CO_2. Cells were grown in RMPI buffered by MOPS in either ambient 37 C or 5% CO2 shaking at 250 rpm. OD600 was measured using BioTek Epoch microplate reader every 24 hours. Each strain was compared via 1 way ANOVA where $\alpha = 0.05$. None of the ambient or 5% CO_2 strains were found to be statistically significant within each specific trial. $p = 0.9981$ and $p = 0.9918$ respectively.


A
Percent survival
Days Post Infection
Untouched
PBS
HK
KN99a
Δato1
Δato2
Δato3
Δato12
Δato13
Δato23
Δato123


B
Percent survival
Day Post Infection
Untouched
KN99a
Δato1
Δato2
Δato12

C
Percent survival
Day Post Infection
Untouched
KN99a
Δato1
Δato3
Δato13


D
Percent survival
Day Post Infection
Untouched
KN99a
Δato2
Δato3
Δato23

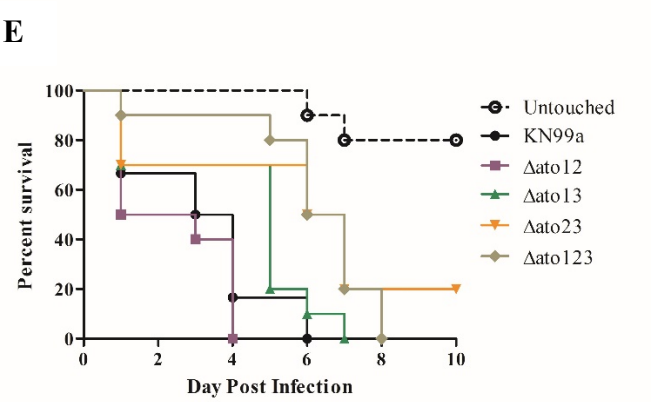

E
Percent survival
Day Post Infection
Untouched
KN99a
Δato12
Δato13
Δato23
Δato123

Figure 2.10: Mutants missing *ATO2* and *ATO3* show virulence in an invertebrate model compared to wild type. *Galleria mellonella* were injected and kept at 37ºC. Surviving larvae were counted over ten days. Controls of untouched Galleria, PBS injected, and heat-killed wild type injected were used. A log-rank (Mantel-Cox) test indicates that curves are significantly different from each other. Chi square = 39.02. df = 10. $P < 0.0001$.

Supplemental Data

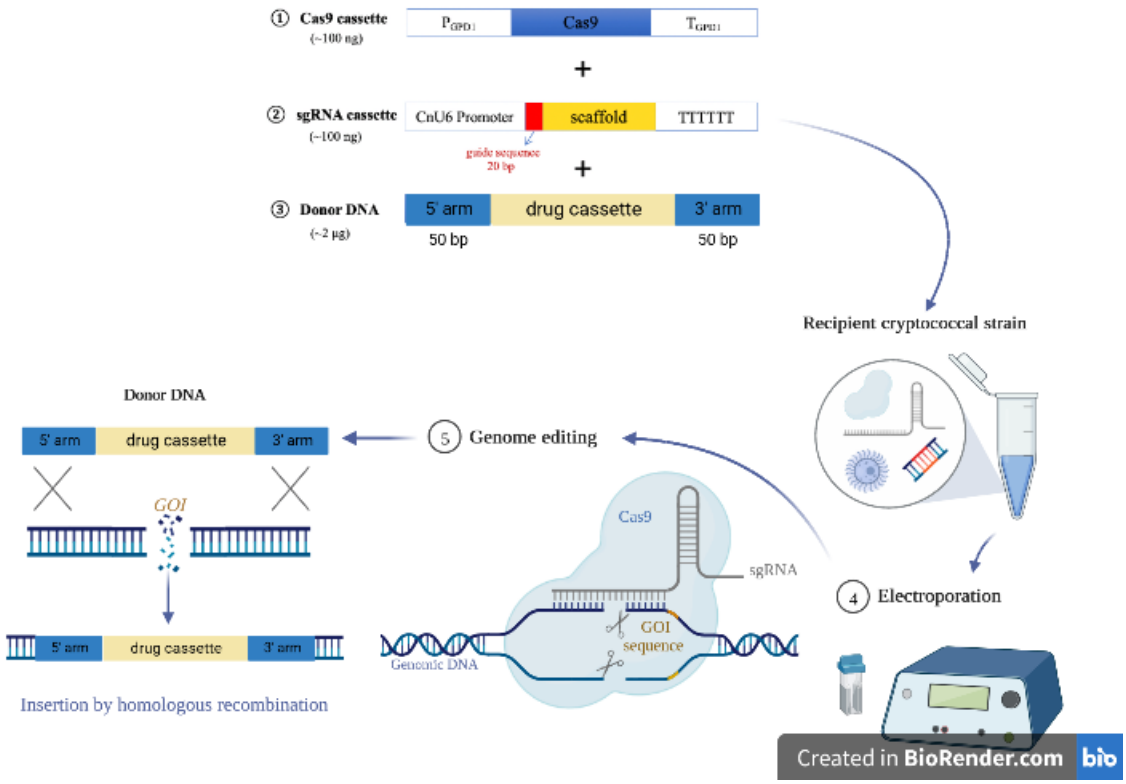
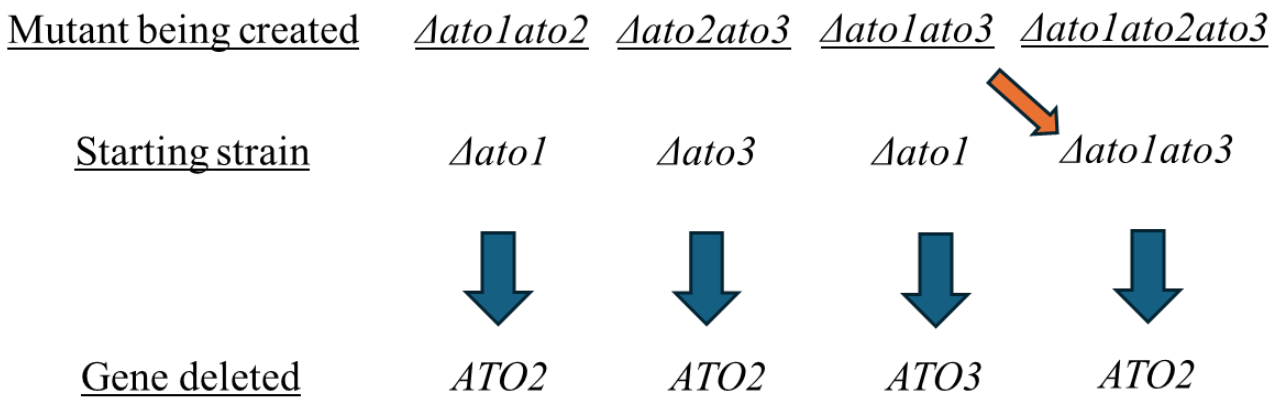
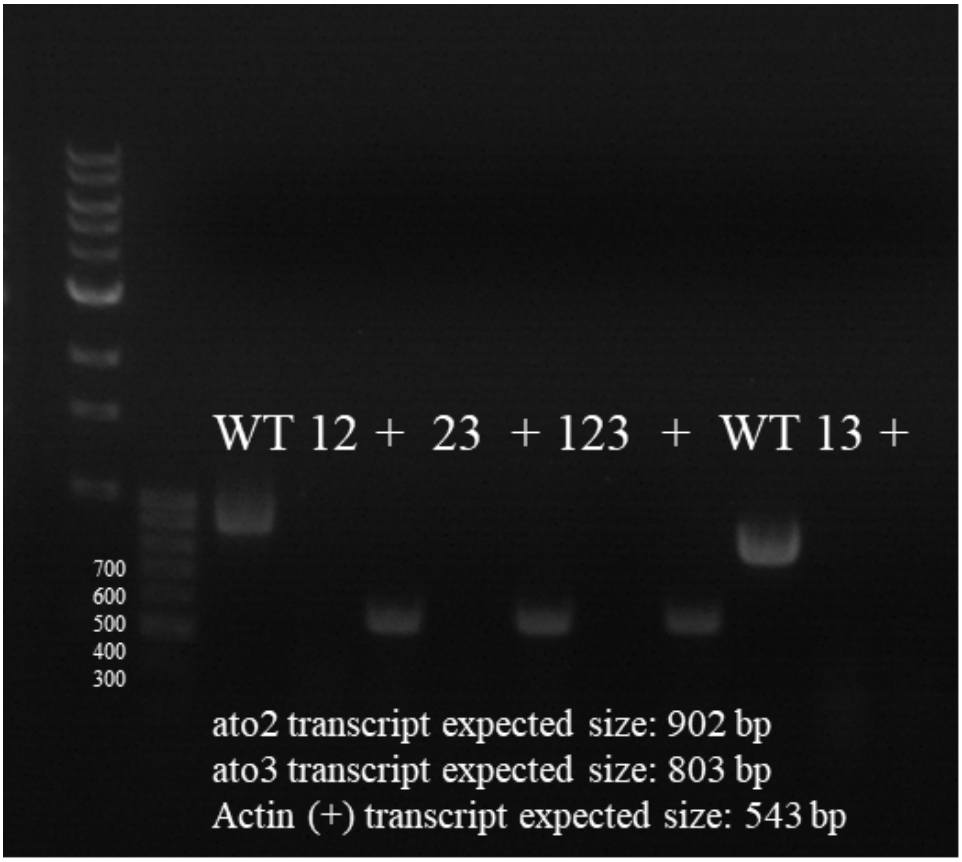


Figure 2.1 Transient CRISPR-Cas9 Coupled with Electroporation (TRACE).

Adapted from Lin et al. Made with BioRender by Dr. Perry Kezh.



Supplemental Figure 2.2 Mutant Creation Workflow. Starting strains from the Madhani library were used to generate double mutants and a triple mutant. The *Δato1ato3* double mutant generated was used to generate the *Δato1ato2ato3* triple mutant.



Supplemental Figure 2.2 RT-PCR Qualification of Transformants. Gel image of RT-PCR of transformed strain RNA. Lanes (left to right): 1. 1 kb ladder 2. 100 bp ladder 3. Wild type RNA (ato2 RT primers) 4. *Δato1ato2* RNA (ato2 RT primers) 5. *Δato1ato2* RNA (actin primers) 6. *Δato2ato3* RNA (ato2 RT primers) 7. *Δato2ato3* RNA (actin primers) 8. *Δato1ato2ato3* RNA (ato2 RT primers) 9. *Δato1ato2ato3* (actin primers) 10. Wild type RNA (ato3 RT primers) 11. *Δato1ato3* RNA (ato3 RT primers) 12. *Δato1ato3* RNA (actin primers). *ATO2* transcript expected size: 902 bp. *ATO3* transcript expected size: 803 bp. Actin transcript expected size: 543 bp. Note that the *Δato1ato3* RNA did not produce a positive actin control, indicating that there is likely an issue with the RNA and/or the strain itself.

CHAPTER THREE

FUTURE DIRECTIONS

Will Betsill and Kerry Smith

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Further investigation of the ATO family in C. *neoformans* is required to fully understand the role of these transporters. We were able to generate double and triple mutants using CRISPR-Cas9. We reaffirmed previous results that found that *Δato1* had a growth defect on acetate, supporting the idea that Ato1 is an acetate transporter. We found that *ato* single mutants grew similarly to a wild type strain on other alternative carbon sources. We found that mutant strains missing *ATO3* exhibited a resistance to SDS. *ato* mutants were found to show variable growth on pH plates and liquid media. We found that these mutants also produce visible melanin sooner than the wildtype. We found that *ato* mutants grew normally in 5% CO_2. Lastly, we found that mutants missing *ATO2* and *ATO3* show strongest survival in an invertebrate model.

We have aligned the amino acid sequences encode ATO genes to compare conserved residues and identify key residues using TCoffee (**Figure 3.1**). In a similar fashion, we created several theoretical phylogenies comparing Ato proteins across related fungi using MEGA11 (MEGA) (**Figure 3.2**) This has allowed us to develop a greater understanding of which residues may be important in acetate transport. Comparing amino acid sequences across other fungi has also given us clues as to the function of the proteins.

We have modeled each Ato using AlphaFold (Google) to examine the predicted structure of each protein (**Figure 3.3**). We found that each prediction had a common alpha helix motif with a low prediction confidence strand coming off the main structure. If these structures exist in these proteins, it is possible that these structures are binding sites for substrates involved in signaling.

Various biochemical techniques could be used to disclose the structure and function of ATO family proteins. For example, these proteins could be isolated for the purpose of solving the crystal structure. Knowing the structure could also reveal which amino acid residues are playing functional roles. CRISPR-Cas could also be a valuable tool for examining key residues. To understand function, we could alter certain residues and then reinsert the altered gene back into the Safe Haven 2 region to assess if the altered copy could complement the deletion. We could also reexamine experiments where we saw a phenotype to see if the altered residue was related to the phenotype. This would pair well with experiments measuring the uptake of radiolabeled acetate. Previous work in our lab found that Ato1 is essential for acetate uptake and that both Ato1 and Ato2 are essential for acetate uptake in carbon source starvation conditions using C^{14} acetate (Grace Kisirkoi and Kerry Smith, unpublished). Similar experiments could be repeated with other metabolites that Ato members are suspected of transporting such as Lactate. This would provide clear evidence that certain metabolites are brought into the cell or not when Ato transporters are present. Following this, mCherry tagging would prove useful to assess localization of Ato proteins to confirm predictions that these proteins are localized at the plasma membrane. The primary caveat is that tagging occasionally affects localization.

Alternatively, we could further examine ammonium export which has been determined to be the function of Ato proteins in *C. albicans*.

To understand what role(s) these transporters play when C. *neoformans* is phagocytosed by macrophages, a macrophage survival assay should be performed. This experiment would provide information about whether macrophages are able to survive ingesting *ato* mutants as well as if these mutants themselves survive in the phagolysosome less well than the wildtype strain. These experiments link well to our pH experiments in deciphering how *C. neoformans* regulates the phagolysosome to reduce ROS stress.

As previously discussed, G. *mallonella* are often used for survival assays due to their simple innate immune system and use in bacteria virulence modelling. While this experiment was performed at 37°C due to this temperature mimicking typical host temperature, this experiment should be repeated but at 30°C since C. *neoformans* mutants often show different growth patterns at different temperatures. Most notably, *Δato3*, *Δato2ato3*, *Δato1ato2ato3* mutants show strong resistance to growth in the presence of SDS at both temperatures whereas other mutants and the wildtype struggle to grow at 37°C, but not 30°C. We also observe *Δato3*, *Δato1ato2*, and *Δato1ato3* mutants grow better on 2% acetate YNB-AA (pH 6) at 37°C rather than 30°C. This would provide us with a wider insight into how mutants behave when confronted *in vivo*.

Mice represent a more complex model to examine virulence but bring researchers closer to an environment similar to humans. Previous studies have found conflicting results regarding survival of Galleria and mice when inoculated with *ato* mutants (Charles Specht, Grace Kisirkoi, Kerry Smith, and Stuart Levitz, unpublished). The *Δato1* and *Δato1ato2*

mutants of these studies showed survival differences in Galleria and mice. Larvae injected with *Δato1* survived, while mice did not. However, both Galleria and mice survived when injected with *Δato1ato2* fungi, indicating importance in virulence. Counting colony forming units in murine lungs would also be useful in establishing a timeline of infection since we would expect a continuous growth in CFUs, but previous studies found that some mutants display a drop and then recovery in fungal load over time. Repeating these experiments in full would provide useful information regarding virulence in the lungs.

Lastly, a speculative model was created to show how the Atos may be functioning (**Figure 3.4**). Ato1 has certainly been shown to play an important role in acetate transportation. Ato2 is thought to be an acetate sensor the regulates ammonia exportation by Ato3 in acidic environments. Further experiments will have to be undertaken to examine these theories.

Figures

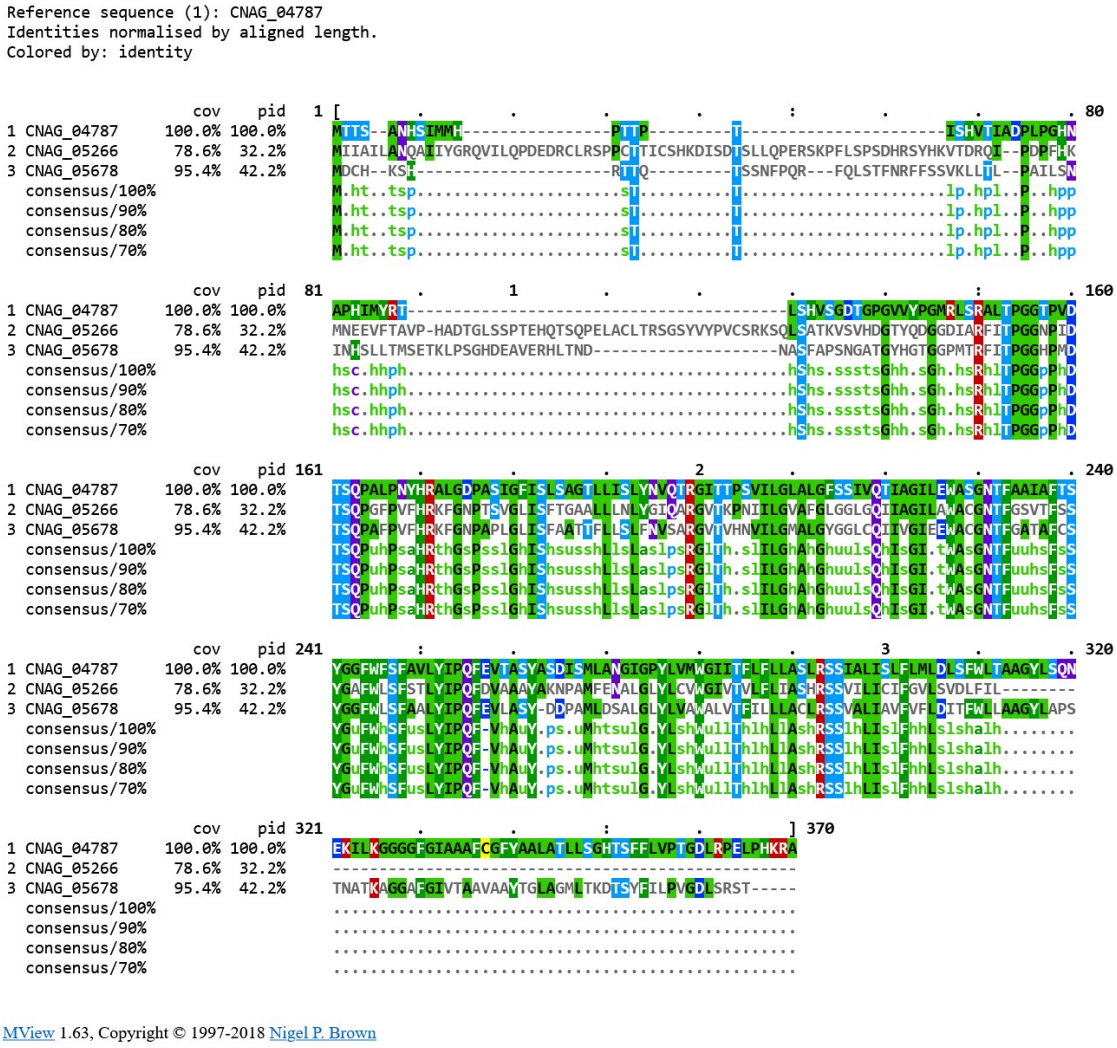

Figure 3.1: Ato Residue Sequence Alignment. Residues were compared using a multisequence alignment via TCoffee. Ato1 is CNAG_04787. Ato2 is CNAG_05266. Ato3 is CNAG_05678.

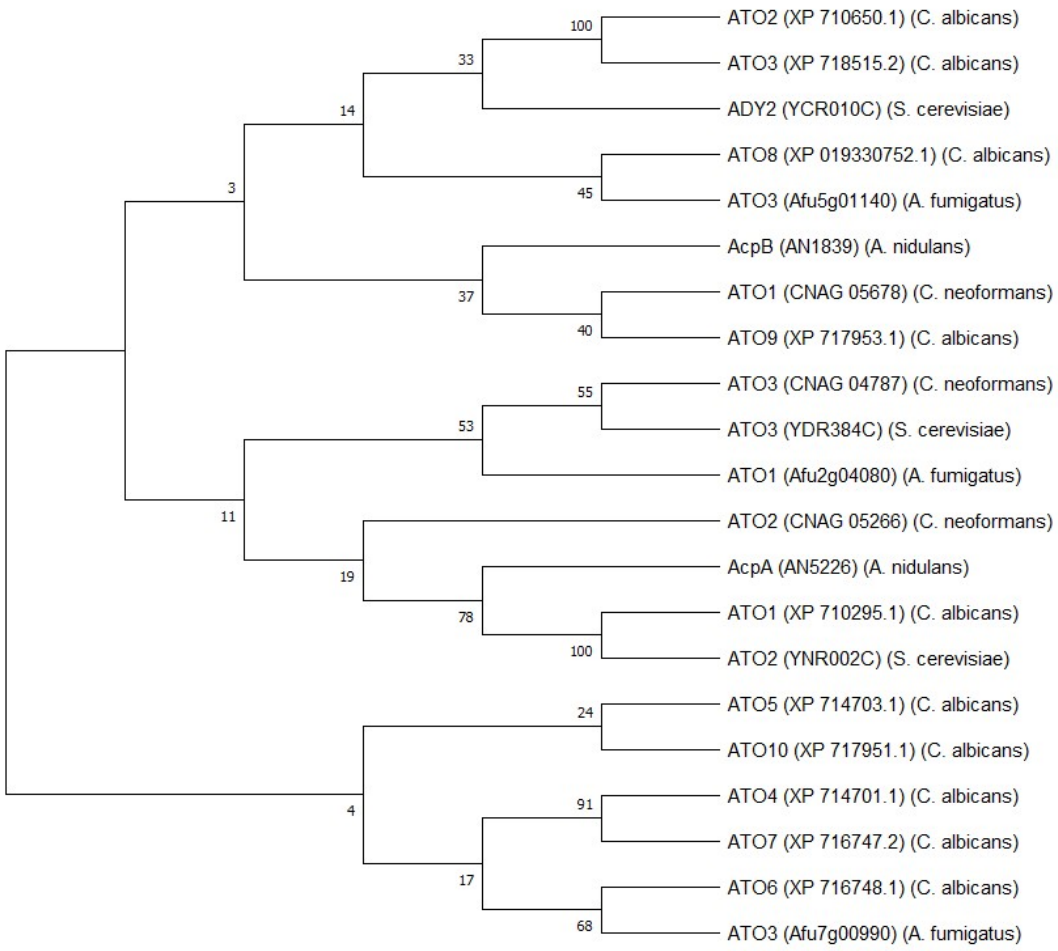

Figure 3.2: Predicted phylogeny of fungal Ato transporters. Sequences were compared using neighbor joining at 1000 bootstraps in MEGA11 (MEGA). The percentage of replicate trees associating taxa together are shown next to the nodes. Gamma distance a = 2.

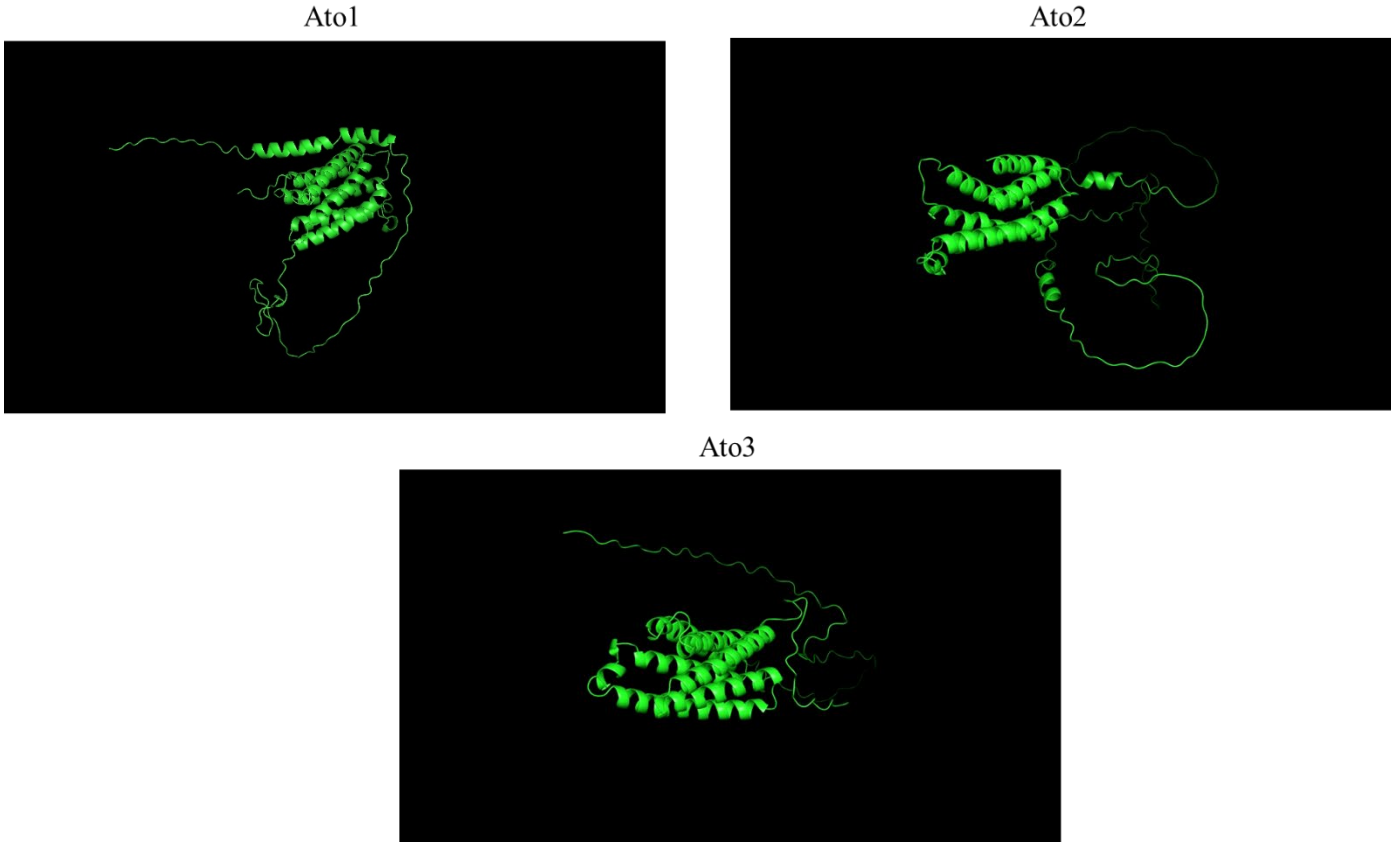

Figure 3.3: Ato protein prediction models. Prediction models were created using AlphaFold (Google). Prediction models were visualized using PyMOL (Schrödinger, Inc).

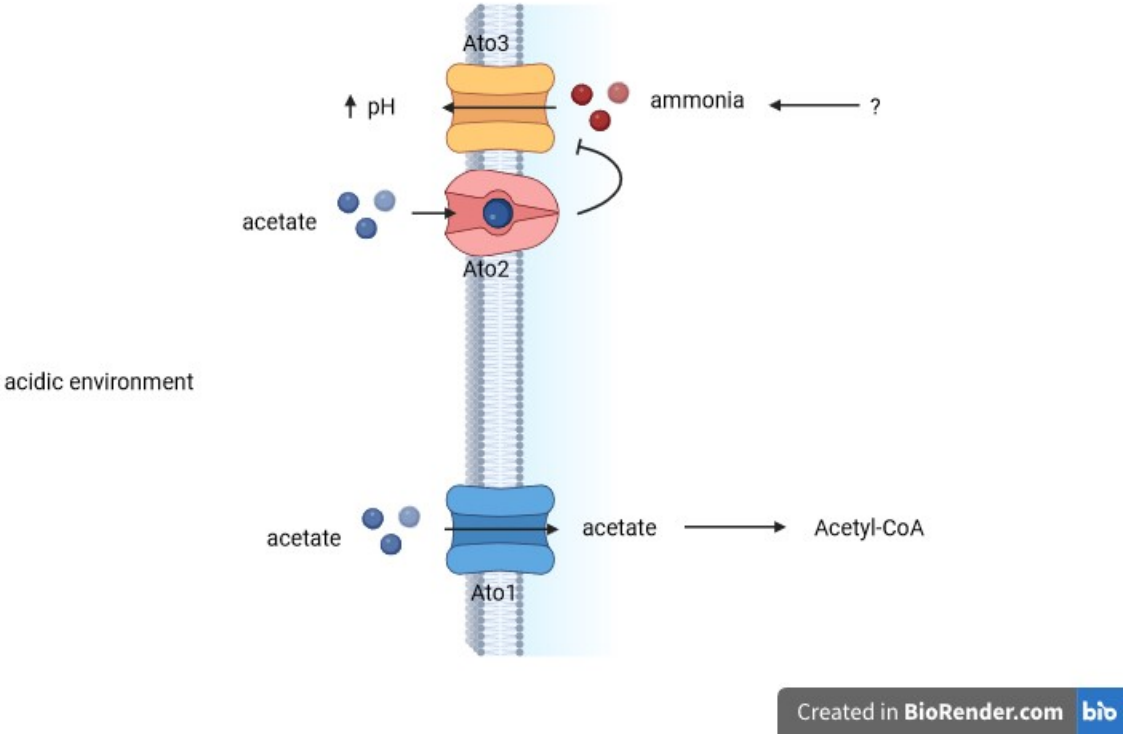

Figure 3.4: Predicted Ato Mechanisms. Ato1 is thought to transport acetate into the cell for utilization. Ato2 is thought to be an acetate sensor the regulates ammonia exportation by Ato3 in acidic environments. Made in BioRender.