The Impact of Nitrogen Limitation on Virulence and the Quorum Sensing Response in *Cryptococcus neoformans*

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Thesis presented in partial fulfilment of the requirements for the degree of Master of Science in the Faculty of Science at Stellenbosch University

Date: March 2020

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DECLARATION

By submitting this thesis electronically, I declare that the entirety of the work contained therein is my own, original work, that I am the sole author thereof (save to the extent explicitly otherwise stated), that reproduction and publication thereof by Stellenbosch University will not infringe any third party rights and that I have not previously in its entirety or in part submitted it for obtaining any qualification.

March 2020

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SUMMARY

Cryptococcus neoformans may cause life-threatening meningitis in immune-compromised populations, with high mortality rates despite measures currently put in place to treat infections. It is, therefore, critical that new and effective anticryptococcal drugs and treatment strategies are developed. Using an antipathogenic approach, which does not directly kill the yeast but rather targets key virulence factors or quorum sensing (QS), is a promising, novel strategy. However, the regulation of cryptococcal QS, and how this process influences virulence factor expression, has not been characterised. In particular, the dynamics of these processes under nitrogen-limiting conditions, which the pathogen experiences in its natural environment and during host infection, has not been defined. This study aimed to characterise the expression of cryptococcal virulence factors during nitrogen limitation (NL) and whether this was being influenced by QS. Furthermore, this study aimed to engineer a *C. neoformans* QS biosensor to monitor QS responses during NL and to screen for potential compounds that could interfere with cryptococcal QS.

After growth in nitrogen-limiting conditions relevant to the *Cryptococcus* natural niche, *C. neoformans* H99 displayed a thicker polysaccharide capsule, as well as higher urease and laccase activities. Melanisation and capsule thickness were specifically increased during both NL and temperature stress. Ergosterol biosynthesis also appeared to be induced during NL and temperature stress. A *C. neoformans* CK0289 QS mutant also expressed thicker capsules and increased urease activity during NL. However, capsule thickness, together with melanisation and ergosterol biosynthesis, were expressed notably weaker than *C. neoformans* H99. This suggests that NL does influence the virulence phenotype, but that QS may be required to signal for increased virulence factor and cell membrane ergosterol production during NL.

Garlic-derived organosulfides and synthetic derivatives thereof were selected as compounds to screen for potential anti-QS activity against *C. neoformans.* The natural organosulfides showed potent antifungal activity and affected the expression of *C. neoformans* virulence factors. Specifically, capsule thickness was reduced and melanisation completely inhibited. Despite having no inhibitory effect on urease activity or ergosterol biosynthesis, these compounds still serve as possible candidates for the development of antipathogenic drugs against *C. neoformans*. To determine whether these compounds have quorum quenching activity, a biosensor responsive to cryptococcal QS and able to monitor changes in this process was designed. Although the biosensor was successfully constructed in *Saccharomyces cerevisiae*, no signal could be detected.

This study has demonstrated that NL is an important signal that influences the virulence of *C. neoformans*, and that this relationship is potentially affected by QS. Further research is needed to comprehensively characterise this phenomenon as this would provide valuable information in understanding the physiology and pathogenicity of *C. neoformans*.

OPSOMMING

Ondanks maatreëls tans in plek om infeksies te behandel kan *Cryptococcus neoformans* lewensgevaarlike meningitis in immuun-gekompromitteerde populasies veroorsaak met hoë sterftesyfers. Daarom is dit krities om nuwe en effektiewe antikriptokokkale middels en behandelingstrategieë te ontwikkel. ‘n Belowende en ongewone strategie is die antipatogeniese benadering wat sleutel virulensie faktore of kworumwaarneming (KW) eerder as direkte gisdoding teiken. Die regulering van kriptokokkale KW en hoe die proses die uitdrukking van virulensie faktor affekteer is egter nog onbekend. Die dinamika tussen die prosesse onder stikstofbeperkende kondisies, soos wat die patogeen in sy natuurlike omgewing en tydens gasheerinfeksie ondervind, is veral nog nie omskryf nie. Hierdie studie stel ten doel om die uitdrukking van kriptokokkale virulensie faktore tydens stikstofbeperking (SB) te karakteriseer en die rol wat KW speel te ondersoek. Die studie beoog verder ook om ‘n *C. neoformans* KW biosensor te ontwikkel om KW reaksies tydens SB te kan meet en die indentifisering van potensiële kriptokokkale KW inmenging verbindings moontlik te maak.

Tydens groei onder stikstofbeperkende kondisies, wat die natuurlike nis van *Cryptococcus* emuleer, vertoon *C. neoformans* H99 ‘n vergrote polisakkariedkapsule, asook hoër urease en lakkase ensiemaktiwiteit. Melanienvorming en kapsulegrootte word veral verhoog tydens beide SB en temperatuurstres. Ergosterolbiosintese word ook spesifiek geïnduseer tydens SB en temperatuurstres. Vergrote kapsule en verhoogte urease aktiwiteit word ook onder SB kondisies deur die *C. neoformans* CK0289 KW mutant vertoon, maar kapsulegrootte, melanienvorming en ergosterolbiosintese word beduidend swakker in vergelyking met *C. neoformans* H99 uitgedruk. Die resultate dui daarop dat SB die virulensie fenotiepe beïnvloed, en dat KW die nodige sein vir verhoogte virulensie faktore en selmembraan ergosterol produksie tydens SB verskaf.

Knoffel-organosulfide en sintetiese derivate is geselekteer om vir potensiele anti-KW verbindings teen *C. neoformans* te sif. Die natuurlike organosulfide het kragtige swamdodende aktiwiteit vertoon en het die uitdrukking van *C. neoformans* virulensie fakore beïnvloed. Kapsulegrootte is spesifiek verlaag en melanienvorming volledig geïnhibeer. Hierdie verbindings dien steeds as moontlike antipatogeniese kandidaatmiddels teen *C. neoformans*, ten spyte van die afwesigheid van urease ensiemaktiwiteit en ergosterolbiosintese onderdrukking. ’n Kriptokokkale KW-sensitiewe biosensor om die kworumsmorings aktiwiteit van die verbindings asook verandering in die KW proses te bepaal is ontwerp. Alhoewel die biosensor suksesvol in *Saccharomyces cerevisiae* gebou is, kon geen sein waargeneem word nie.

Die studie demonstreer die belangrike rol van SB op *C. neoformans* virulensie faktore, en dat KW hierdie verhouding affekteer. Verdere navorsing word benodig om die fenomeen volledig te

karakteriseer, om waardevolle inligting en ’n dieper verstaan van die fisiologie en patogenisiteit van *C. neoformans* te bevorder.

ACKNOWLEDGEMENTS

I would like to thank the following people for their contribution throughout this study:

Dr. Volschenk for his continued support and valuable insight throughout the course of this project.

Kim for sharing her intellect, for her patience during all my rookie mistakes, and for teaching me valuable skills throughout the past few years.

Shelly and Shauni for sharing their expertise in cloning and yeast transformations, respectively, during the many stressful periods of troubleshooting.

Prof. Botha, Caylin, CJ and Jo-Marie for their input during the early stages of this project and their continued advice throughout.

Dr. Catherine Kaschula, Doaa and Dan for kindly providing the organosulfur compounds used in this study, as well as for sharing their knowledge in organic chemistry.

Prof. Hiten Madhani, Sandra and Beiduo for not only providing us with the *Cryptococcus neoformans* mutant used in this study but also for sparing valuable time helping with *Cryptococcus* transformations.

Riaan, Gerhardt, Lauren, Jerard and Jeanie for their input and assistance.

Elana and everyone else from the Wolfaardt lab for being kind enough to allow me to use their biosafety cabinet for prolonged periods of time.

Everyone else from the Department of Microbiology for their support, assistance and helpful advice.

The National Research Foundation for funding this project.

A special thanks to Marisa, Zoë, Thoreefa and my sisters for all the interesting conversations, being great friends and for keeping me sane.

And to the Universe, thanks.

DECLARATION BY THE CANDIDATE

This dissertation is presented as a compilation of six chapters, where each chapter is introduced separately. The development and writing of the chapters were my principal responsibility. Where this is not the case, a breakdown is included indicating the nature and extent of the contributions of co-authors.

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The undersigned hereby confirm that:

1. the declaration above accurately reflects the nature and extent of the contributions of the candidate and the co-authors to Chapters 1 to 6,
2. no other authors contributed to Chapters 1 to 6, besides those specified above, and
3. potential conflicts of interest have been revealed to all interested parties and that the necessary arrangements have been made to use the material in Chapters 1 to 6 of this dissertation.

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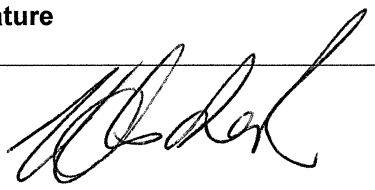	Stellenbosch University	
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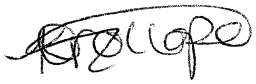	Stellenbosch University	
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CHAPTER 1

General introduction and project aims

Chapter 1: General introduction and project aims

1.1 General introduction

The encapsulated basidiomycetous yeast, *Cryptococcus neoformans*, is a facultative intracellular pathogen that can cause cryptococcosis in immune-compromised mammalian hosts (Buchanan and Murphy, 1998). Initially, cryptococcal infections in humans present mild flu-like symptoms that later develop into pneumonia and eventually meningitis (Saag et al., 2000; Perfect and Bicanic, 2015). Infection generally occurs after airborne basidiospores are inhaled and deposited in the lower respiratory tract (Buchanan and Murphy, 1998). Cryptococcosis is regarded as the fifth most lethal infectious disease in the world (World Health Organisation, 2018) and is responsible for a mortality of 180000 annual deaths, of which over 130000 are reported in sub-Saharan Africa alone (Brodt et al., 1997; Offiah and Naseer, 2016; Rajasingham et al., 2017).

Numerous virulence factors attribute to the ability of *C. neoformans* to cause infection (Almeida et al., 2015; Malachowski et al., 2016). The most well-studied virulence factors of *C. neoformans* include the polysaccharide capsule, the deposition of melanin in its cell wall, phospholipase and urease activity, as well as its ability to survive at host physiological temperature (Bulmer et al., 1967; Heere et al., 1975; Wang et al., 1996; Cox et al., 2000; Cox et al., 2001). In most microbial pathogens, the expression of virulence factors is controlled by a cell density-dependent response mechanism termed quorum sensing (QS) (Bandara et al., 2012). This process involves the production, release and detection of small signalling molecules (autoinducers) that, after reaching a threshold concentration during high cell densities, induce a highly coordinated change in population dynamics and gene expression patterns. A small peptide, called Qsp1 (QS-like peptide 1), mediates QS in *C. neoformans* (Lee et al., 2007), which affects the expression of over 1700 genes and influences the pathogen's virulence phenotype (Homer et al., 2016). To date, only a few studies have investigated the role Qsp1 plays in regulating virulence and other physiological characteristics of *C. neoformans* (Lee et al., 2009; Albuquerque et al., 2014; Homer et al., 2016; Tian et al., 2018; Trevijano-Contador et al., 2018; Camacho et al., 2019). Thus, changes in cryptococcal QS in response to various nutrient and growth conditions, and how this affects virulence, have not been explicitly investigated.

In the environment, *C. neoformans* is usually isolated from the woody substrate of trees, pigeon guano and soil (Emmons, 1951; Emmons, 1955; Gibson and Johnston, 2015). Here the yeast is constantly faced with temperature fluctuations and limiting concentrations of accessible or preferred nutrient sources, such as nitrogen. Similarly, during mammalian host infection, *C. neoformans* is exposed to physiological temperature (37°C) and nitrogen-poor conditions where it must metabolise alternative nitrogen sources in order to survive (Ries et al., 2018). Nitrogen metabolism in fungi is tightly regulated by nitrogen catabolite repression (NCR), a process that represses the expression

of genes that encode for the catabolism and transport of alternative nitrogen sources in the presence of preferred nitrogen sources (Marzluf, 1997). Thus, alternative nitrogenous compounds are only imported and metabolised by the cell during conditions of nitrogen limitation (NL), where preferred nitrogen sources in the cell's vicinity are in short supply or absent. Previously, NL has been demonstrated to affect ergosterol biosynthesis and virulence factor expression in *C. neoformans* (Lee et al., 2011; Bosch et al., unpublished). Although QS via Qsp1 was found to be important for virulence in *C. neoformans* (Homer et al., 2016), the effect of NL on QS, and how this would affect virulence factor expression, is still unknown.

Current anticryptococcal treatment strategies include the use of drugs such as fluconazole (FLC), amphotericin B (AmB) and flucytosine. When used in monotherapy, FLC is highly ineffective due to its fungistatic nature (Larsen et al., 1990; Schaars et al., 2006), but is recommended for use in combination with AmB and flucytosine (Perfect et al., 2010; Smith et al., 2015). A recent clinical trial demonstrated that AmB and flucytosine combination therapy can clear cryptococcal infections in human hosts (Molloy et al., 2018). However, AmB is expensive to administer and is cytotoxic (Perfect and Bicanic, 2015), while flucytosine is largely unavailable in sub-Saharan Africa where *C. neoformans* infections are the most prominent (Perfect and Bicanic, 2015; Azevedo et al., 2016). Furthermore, *C. neoformans* has been reported to acquire resistance to these drugs after long-term use (Gago et al., 2016). This highlights the requirement for the development of novel anticryptococcal treatment solutions.

A promising alternative treatment strategy involves the use of drugs that target cellular components not important for growth and survival, such as those that are involved in virulence and QS. This is referred to as the antivirulent or antipathogenic approach (Fong et al., 2017). An advantage to using this approach is that it reduces the likelihood of an organism acquiring drug resistance as it promotes a weaker selection pressure compared to drugs that target essential cellular components (Cegelsky et al., 2008; Jakobsen et al., 2012; LaSarre and Federle, 2013; Kirienko et al., 2019). To date, no studies have tested the potential of various drugs in inhibiting QS in *C. neoformans*. However, some studies have reported on the capacity of medicinal plant-derived compounds and extracts in inhibiting the expression of *C. neoformans* virulence factors (Cardoso et al., 2017; Kumari et al., 2017; Samie et al., 2019). Because virulence factor expression is regulated by QS (Bandara et al., 2012), this suggests that QS may have been affected and thus motivates for the exploration of medicinal plants as alternative sources for potential quorum quenching drugs against *C. neoformans*. Previously, garlic (*Allium sativum* L.) extracts, as well as natural and synthetic garlic organosulfur compounds, have shown potent antipathogenic activity against bacteria, inhibiting both virulence factor expression and QS (Jakobsen et al., 2012; Fong et al., 2017; Li et al., 2018; Li et al., 2019). Similarly, natural garlic compounds and crude extracts have displayed antifungal activity against *C. neoformans* (Yamada and Azuma, 1977; Fromtling and Bulmer, 1978; Davis et al., 1990;

Khan and Katiyar, 2000; Rehman and Mairaj, 2013). Therefore, garlic may serve as a promising candidate for alternative anticryptococcal drug discovery.

Altogether, understanding the interplay between virulence factor expression and QS, as well as the effect NL has on these factors, may provide further insight into the physiology and pathogenesis of *C. neoformans*. This may also aid in the development of novel cryptococcal treatment solutions and direct alternative strategies in anticryptococcal drug design.

1.2 Aims and objectives

1.2.1 Aims of this study

- To examine the effect NL has on virulence factor expression in *C. neoformans* and whether this can be linked to QS.
- To screen for compounds that could potentially interfere with the cryptococcal QS response and inhibit virulence factor expression.
- To construct a yeast biosensor that is able to monitor *C. neoformans* QS via Qsp1.

1.2.2 Study objectives

- To quantitatively measure changes in the thickness of the *C. neoformans* capsule, cell membrane ergosterol content, melanisation and its urease activity in response to growth in nitrogen-limiting media (NLM).
- To compare differences in cell membrane ergosterol content and the virulence phenotype of a *C. neoformans* H99 wild type strain and a *C. neoformans* CK0289 QS mutant strain during NL.
- To screen garlic-derived organosulfur compounds for their potential to inhibit ergosterol biosynthesis, virulence factor expression and QS.
- To design a yeast QS biosensor that can respond to *C. neoformans* Qsp1 and measure changes in this process.

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CHAPTER 2

LITERATURE REVIEW

Quorum sensing in *Cryptococcus neoformans*: a potential antipathogenic target

Chapter 2: Quorum sensing in *Cryptococcus neoformans*: a potential antipathogenic target

2.1 *Cryptococcus neoformans* and cryptococcosis

Invasive fungal infections caused by the dimorphic basidiomycetous yeast, *Cryptococcus neoformans*, can lead to the development of life-threatening cryptococcosis. Cryptococcosis usually manifests after airborne spores or desiccated yeast cells are inhaled (Buchanan and Murphy, 1998), and results in the development of pneumonia and eventually meningitis (Saag et al., 2000; Perfect and Bicanic, 2015). Meningitis develops as a consequence of the yeast spreading from the lungs into the bloodstream, and finally to the brain (Santiago-Tirado et al., 2017), where cells become lodged between brain tissue and cause severe inflammation and swelling thereof. Individuals susceptible to disseminated cryptococcal infections include those with compromised immune systems, especially people with autoimmune diseases (Lin et al., 2015b), those infected with the Human Immunodeficiency Virus (HIV), or patients undergoing organ transplant surgery and chemotherapy (Coelho et al., 2014). Although immune-competent individuals have been reported to be infected by the sister species, *C. gattii* (Sorrell, 2001), the majority of reported cryptococcal infections have been associated with serotype A strains of *C. neoformans* (*C. neoformans var. grubii*) (Datta et al., 2009). *Cryptococcus* spp. are ubiquitous, found in both environmental and urban settings, particularly in association with the soil, decaying substrates of trees and pigeon excrement (Emmons, 1951; Emmons, 1955; Gibson and Johnston, 2015). Because of this broad ecological niche, most individuals have likely already been exposed to *C. neoformans* soon after birth (Goldman et al., 2001).

Cryptococcosis is the fifth most lethal infectious disease in the world (World Health Organisation (WHO) 2018), following acquired immunodeficiency syndrome (AIDS), tuberculosis, malaria and diarrhoea. Cryptococcal meningitis is responsible for a global mortality of 180000 deaths per year, of which 75% occurs in sub-Saharan Africa alone (Brodt et al., 1997; Offiah and Naseer, 2016; Rajasingham et al., 2017). Although a 45% decrease in cryptococcosis-induced mortality in AIDS patients has been reported after the implementation of antiretroviral therapy (Park et al., 2009), up to 60% of treated patients still die to cryptococcal meningitis (Jarvis and Harrison, 2007; Armstrong-James et al., 2014). Moreover, around 15% of all annual AIDS-related deaths are caused by cryptococcosis (Park et al., 2009; Rajasingham et al., 2017). Despite this, cryptococcosis is still not recognised as a neglected tropical disease by the WHO (Molloy et al., 2017).

2.2 Virulence factors of *C. neoformans* that allow its survival in the lung and dissemination to the brain

The pathogenicity of *C. neoformans* is attributed to its numerous virulence factors (Almeida et al., 2015; Malachowski et al., 2016). Virulence factors are biosynthetic products produced by an organism, or certain physiological characteristics, that not only improve its fitness and survival in harsh environments but are often also associated with disease progression (Casadevall and Pirofski, 2001; Wu et al., 2008). Only a few of the 150 known cryptococcal virulence factors (Malachowski et al., 2016) have been studied explicitly. These include the production of a polysaccharide capsule (Bulmer et al., 1967; Zaragoza et al., 2009), the deposition of melanin in the cell wall (Wang et al., 1996; Rosas et al., 2000), urease activity (Cox et al., 2000; Olszweski et al., 2004), phospholipase activity (Cox et al., 2001) and the ability to tolerate growth at 37°C (Heere et al., 1975).

The polysaccharide capsule of *C. neoformans* consists of a thick, complex mesh of carbohydrate macromolecules that surrounds the cell body. The main component of the capsule is glucuronoxylomannan (>90%), with galactoxylomannan and mannoproteins found in smaller proportions (Cherniak et al., 1980; Turner et al., 1984). Estimations using multivariate linear regression analysis infer that the polysaccharide capsule contributes to about 25% of the cryptococcal virulence phenotype (McClelland et al., 2006). The capsule can serve as a barrier, protecting cells from desiccation (Aksenov et al., 1973), antimicrobials (Zaragoza et al., 2008), oxidative stress (Zaragoza et al., 2008), predation (Steenbergen et al., 2001) and attack by components of the mammalian host immune system (Kozel et al., 1977; Vecchiarelli et al., 1995).

The cryptococcal laccase enzyme protects cells similarly to that of the polysaccharide capsule. This phenoloxidase enzyme can convert various exogenous phenolic or indolic substrates to melanin (Rhodes et al., 1982; Garcia-Rivera et al., 2005), a brown to black pigment that is deposited in the cell wall. The melanin layer acts as an insoluble barrier and electron scavenger, protecting cells against oxidative stress and heat (Liu et al., 1999; Zaragoza et al., 2008), antifungal drugs (Ikeda et al., 2013) and from growth inhibition induced by various components of the mammalian host immune system (Wang et al., 1995; Liu et al., 1999). Another group of virulence-associated enzymes, ureases, are nitrogen-scavenging enzymes that allow *C. neoformans* to convert urea to a usable nitrogen source in environments with low abundance of favourable nitrogenous compounds. Specifically, the urease enzyme catalyses the one-step conversion of urea to ammonia and carbamate (Cox et al., 2000; Olszewski et al., 2004). Ammonia produced by ureases could potentially damage host tissues (Singh et al., 2013) and may also increase local pH, interfering with the function of immune system components that require an acidic pH for optimal pathogen clearance (Rutherford, 2014).

Although infected mammalian hosts can induce a fever in response to invasive fungal infections (Kluger et al., 1998), where the body temperature is raised in an attempt to clear infections,

C. neoformans strains have been shown to tolerate temperatures up to 43°C (Martinez et al., 2001). Avirulence was observed in mouse and rabbit infection models when *C. neoformans* displayed defects in each of the abovementioned virulence factors (Kwon-Chung et al., 1982; Chang et al., 1996; Odom et al., 1997; Alspaugh et al., 2000; Fu et al., 2018), supporting the importance of these factors in promoting the pathogenesis of *C. neoformans*. Despite this, it is still necessary to study the other remaining cryptococcal virulence factors because avirulence was also observed in strains that show normal capsule, melanin and thermotolerance traits, suggesting that pathogenesis require additional factors (Franzot et al., 1998).

2.3 Quorum sensing

2.3.1 Bacterial quorum sensing

Quorum sensing (QS), a complex chemical communication system used by microorganisms, is well-studied in bacteria with only a few fungi known to be capable of this process. This process is not limited to bacteria and fungi; QS has also been reported for viruses (Erez et al., 2017) and certain animals such as ants (Pratt et al., 2002). Generally, in microorganisms, QS involves the production, release and detection of autoregulatory signalling molecules (autoinducers), which consequently affect population dynamics and gene expression patterns (Bandara et al., 2012). Most microorganisms with studied QS mechanisms are pathogenic. This is because QS usually regulates virulence factor expression and biofilm development, which, in turn, promotes successful infection and protects microorganisms from antimicrobials and the host immune system (Alhede et al., 2009; van Gennip et al., 2009; Chiang et al., 2013). In certain microorganisms, changes in cell morphology, as well as the stimulation of competence and conjugation, are also controlled by QS (Bandara et al., 2012). Other benefits of multicellular growth afforded by QS and biofilm formation include resistance to environmental stressors and predation, as well as gaining better access to available and previously inaccessible nutrients (Lyons and Kolter, 2015).

The first reports of organised density-dependent responses in microorganisms were in 1960 and 1970 for *Streptococcus pneumoniae* and the marine bacterium, *Aliivibrio fischeri*, respectively (Ottolenghi and Hotchkiss, 1960; Nealson et al., 1970). Both bacteria induced specific processes (genetic competence or bioluminescence) only once the cell density increased significantly. These density-dependent processes were later found to be mediated by secreted autoinducers and are now defined as a QS response (Håvarstein et al., 1995; Eberhard et al., 1981).

Bacteria typically use various acyl-homoserine lactones (AHLs), chemically modified peptides or boron-furan-derived chemicals as autoinducers (Verbeke et al., 2017). Peptide-mediated QS signals are usually only employed by Gram-positive bacteria (Taga and Bassler, 2003; Abisado et al., 2018). Initially, autoinducing peptides (AIPs) are produced intracellularly by ribosomes in an immature form and then actively secreted via ATP-binding cassette transporters (Sturme et al., 2002). During and

after secretion, the immature peptide undergoes posttranslational modifications to form the functional, mature peptide. These mature AIPs begin to accumulate in the environment as the density of bacterial cells increase. Once the AIPs reach a threshold concentration, they bind to specific cell-surface receptors, triggering an intracellular signal transduction cascade that eventually activates downstream response regulators (Bassler 1999; Sturme et al., 2002). These regulators then influence the expression of target genes. In some cases, the AIPs themselves are transported back into the cell via cell membrane-bound transporters and directly bind and activate the response regulators (Debunne et al., 2017).

2.3.2 Quorum sensing in eukaryotes

The regulation of density-dependent QS responses controlled by specific compounds in a eukaryote was first implicitly reported for *Candida albicans* (Hornby et al., 2001; Chen et al., 2004). Two alcohols, farnesol and tyrosol, were found to function as regulators of QS-like responses for this yeast. The QS alcohols were shown to be produced by the organism at different levels to control cell morphology, growth and biofilm development. Although QS-like mechanisms have been described for other eukaryotes such as *Saccharomyces cerevisiae* (Chen and Fink, 2006), *Histoplasma capsulatum* (Kugler et al., 2000), *Ceratocystis ulmi* (Hornby et al., 2004), *Neurospora crassa* (Roca et al., 2005), *C. neoformans* (Lee et al., 2007; Homer et al., 2016) and other fungi, the compounds mediating QS have not necessarily been elucidated (Albuquerque and Casadevall, 2012). The most common known compounds implicated in QS that are also shared between yeasts are fusel alcohols such as tyrosol, farnesol, tryptophol, phenethyl alcohol and isoamyl alcohol (Dickinson, 1996; Hornby et al., 2001; Chen and Fink, 2006). These alcohols are generally derived from amino acid catabolism via the Ehrlich pathway (Hazelwood et al., 2008). Although the potential for QS has been implicated in fungi, this mechanism has mostly only been studied and characterised for the ascomycetous yeasts, *C. albicans* and *S. cerevisiae*.

2.4 Quorum sensing in *C. neoformans*

2.4.1 Mechanism of signalling

The opportunistic pathogen, *C. neoformans*, was found to exhibit a cell density-dependent growth effect after the *TUP1* gene, which encodes for a general transcriptional regulator of growth, morphology and mating (Lee et al., 2009), was deleted (Lee et al., 2007). *TUP1* knock-out mutants are unable to grow on solid media at low cell densities. This growth phenomenon was found to be mediated by a short 11 amino acid oligopeptide with an isotopic mass of 1136.23 Da. The peptide sequence (NH_2-NFGAPGGAYPW-COOH) matches the C-terminal region of one of the spliced products of a *C. neoformans* gene previously called *CQS1* (*Cryptococcus* QS-like molecule). Exposure to the peptide (crude, purified or synthetically manufactured) recovered the growth phenotype of *C. neoformans* tup1Δ mutants at low cell densities. Since the peptide appeared to mediate cell density-dependent growth, a characteristic of QS, it was named Qsp1 (QS-like

peptide 1). It was only later where Qsp1 was shown to act directly as a QS molecule (QSM) in *C. neoformans* and, following this, the *CQS1* gene encoding the peptide was renamed to *QSP1* (Homer et al., 2016).

Through transcriptomic analyses, three virulence-associated transcription factors (Gat201, Gat204 and Liv3) were found to be a target of Qsp1. The QS peptide increased the expression of the genes encoding these three transcription factors as well as its own *QSP1* gene (autoregulation), consequently resulting in an increased virulence phenotype. The current model of *Cryptococcus* QS (Fig. 2.1), as proposed by Homer et al. (2016), indicates that Qsp1 is produced intracellularly as an immature propeptide before being processed extracellularly via a membrane-bound peptidase (Pqp1) into mature Qsp1. Only once the peptide is transported back into the cell via an oligopeptide transporter (Opt1) does it induce a QS response. Under saturated conditions, where there are high cell densities, Qsp1 in proximity can be processed and imported more rapidly. Due to autoregulation, this would result in the increased production, release and accumulation of Qsp1, consequently strengthening the QS response and virulence phenotype of the pathogen.

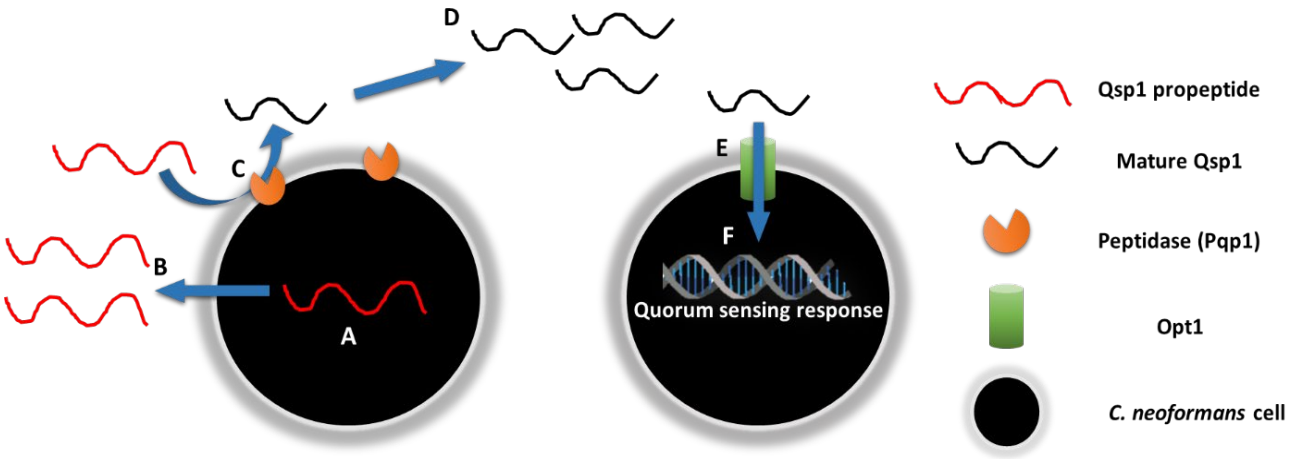

Figure 2.1: Quorum sensing (QS) pathway of *Cryptococcus neoformans*. (A) Immature Qsp1 is produced intracellularly before being released into the extracellular environment (B). (C) Once outside the cell, the pro-region of the immature peptide (Fig. 2.3) is cleaved by a membrane-bound peptidase (Pqp1), and mature Qsp1 is released and accumulates outside the cell (D). (E) Mature Qsp1 is then transported into the cell via an oligopeptide transporter (Opt1). (F) Once imported, mature Qsp1 interacts with intracellular components to control physiological mechanisms generally associated with QS.

2.5 Regulation of virulence by Qsp1

2.5.1 Virulence factor expression

The effect of Qsp1 on the expression of key cryptococcal virulence factors (such as the polysaccharide capsule and melanisation) have, to date, mostly been investigated by comparing phenotypic differences between the *C. neoformans* H99 wild type strain and a *C. neoformans* qsp1Δ mutant strain. Homer et al. (2016) showed that melanisation was variably controlled by Qsp1 and

depended on growth conditions. Additionally, the authors found that there was relatively no difference in capsule thickness between the qsp1Δ mutant and the wild type strain when grown at 37°C. However, at a lower temperature (25°C), the mutant had significantly thicker capsules. The *C. neoformans* QS mutant was exposed to synthetic mature Qsp1 in order to determine whether this would recover the capsule and melanin phenotypes to that of the wild type cells. However, only partial recovery was observed. Previously, Albuquerque and co-workers (2014) reported similar results describing that the QS peptide was not able to fully recover the growth defect of a tup1Δ mutant, but the supernatant of the wild type strain could. This suggests that QS via Qsp1 may not be the sole mechanism regulating virulence and that Qsp1 could be acting in concert with other metabolites produced by *C. neoformans*. This is further supported by the fact that growth conditions influence the effects of purified and synthetic Qsp1. It was suggested that due to the architectural changes in the cell wall and capsule of the *C. neoformans* QS mutant, the binding and import of Qsp1 might be affected and influence the observed phenotype (Albuquerque et al., 2014). Nevertheless, the mutant causes reduced mortality in a mouse model and accumulate to a lower degree in the lungs compared to wild type cells (Homer et al., 2016), indicating that Qsp1-regulated responses are important for virulence and disease progression.

2.5.2 Phagocytosis

During cryptococcal infections, the host's immune system actively mobilises specialised cells, such as macrophages, to eliminate the pathogen (Goldman et al., 2001). Macrophages can recognise antigens on the surfaces of *C. neoformans* cells and engulf them via a process called phagocytosis. Engulfed cryptococcal cells are further enclosed in an acidic phagolysosome within which the cells are bombarded with a cocktail of lytic enzymes and reactive electrophilic compounds (Hünniger and Kurzai, 2018). Usually, this process is sufficient to clear most microbial infections (Mogensen, 2009). In a mouse infection model, Homer et al. (2016) found that virulence was attenuated when *C. neoformans* is incapable of producing Qsp1 (as demonstrated by the *C. neoformans* qsp1Δ mutant strain) – reduced mortality of infected mice was observed compared to wild type cells. The mutant did not display any altered sensitivity to various stressors, such as oxidative or osmotic stress, suggesting that defects in protective stress response mechanisms were likely not responsible for the reduced virulence observed. In contrast to the wild type cells, however, the *C. neoformans* QS mutant did not accumulate to high cell densities within macrophages. At first, this finding would appear to contradict the attenuation of virulence displayed by the qsp1Δ mutant. This is because macrophages are host immune effectors considered necessary for the elimination of infectious propagules and have previously been shown to have the capacity to phagocytose and subsequently kill *C. neoformans* cells (Vecchiarelli et al., 1994), particularly during initial pulmonary infections (Shao et al., 2005). However, an interesting trait of cryptococcal cells is that they can hijack and proliferate within macrophages during infection, potentially facilitating their dissemination throughout the host via the bloodstream (Chrétian et al., 2002; García-Rodas and Zaragoza, 2012). Moreover,

phagocytosed cryptococcal cells grow faster than non-engulfed cells (Diamond and Bennett, 1973) and prefer the acidic pH of phagolysosomes (Levitz et al., 1999). Furthermore, increased dissemination of *C. neoformans* also occurs via dragotcytosis, a process whereby cells are transferred from macrophage to macrophage (Dragotakes et al., 2019).

Various genes involved in cell wall biogenesis and regulation were found to be controlled by Qsp1, and microscopy revealed that qsp1Δ mutant cells presented thinner cell walls than wild type cells (Homer et al., 2016). Therefore, it was hypothesised that the lower rate of phagocytosis experienced by the QS mutant could be due to an altered arrangement of cell wall components, impairing efficient recognition of cell-surface antigens by macrophages (Homer et al., 2016). Consequently, the qsp1Δ mutant cells are not readily phagocytosed and efficiently spread throughout the host, lessening the severity of the infection. Furthermore, phagocytosis may be important for cryptococcal infections because macrophages can serve as an enclosed, saturable environment where cells are exposed to concentrated doses of Qsp1. This would signal for the increased production of armaments, such as the polysaccharide capsule and cell wall melanin, which improve the survival and proliferation of these cells during host phagocytic stress.

2.5.3 Titanisation

Interestingly, the role of Qsp1 has been implicated in the titanisation of cryptococcal cells (Hommel et al., 2018; Trevijano-Contador et al., 2018). *Cryptococcus* usually exists in a heterogeneous population where cell sizes range from 5-7 μm in diameter. An increase in heterogeneity is observed during host infection where a small subset of the population (~20%) can transition into titan cells that span 10-100 μm in diameter (Okagaki et al., 2010) (Fig. 2.2). These aberrantly large cells are hypervirulent (Crabtree et al., 2012), display strong resistance against antifungal drugs, oxidative and nitrosative stress (Gerstein et al., 2015), and can evade the host immune response as they cannot be phagocytosed by macrophages (Okagaki et al., 2012). Furthermore, the progeny of actively replicating titan cells, despite being normal-sized daughter cells, also display increased resistance to stress (Gerstein et al., 2015). Hommel and co-workers (2018) showed that Qsp1 negatively regulates titanisation – the addition of the QS peptide to wild type cells reduced the proportion of titan cells relative to an untreated control. This reduction in titan cell population was also observed when cells were grown in minimal liquid media at a high initial cell density, typical of when high concentrations of Qsp1 would be produced. Further confirming these results, the *C. neoformans* qsp1Δ mutant strain displayed higher titan cell numbers compared to the wild type strain. Using different nutrient-limiting conditions, Trevijano-Contador et al. (2018) also observed the negative regulation of titanisation by Qsp1. They noticed the same effect when cells were exposed to the supernatant of wild type cells.

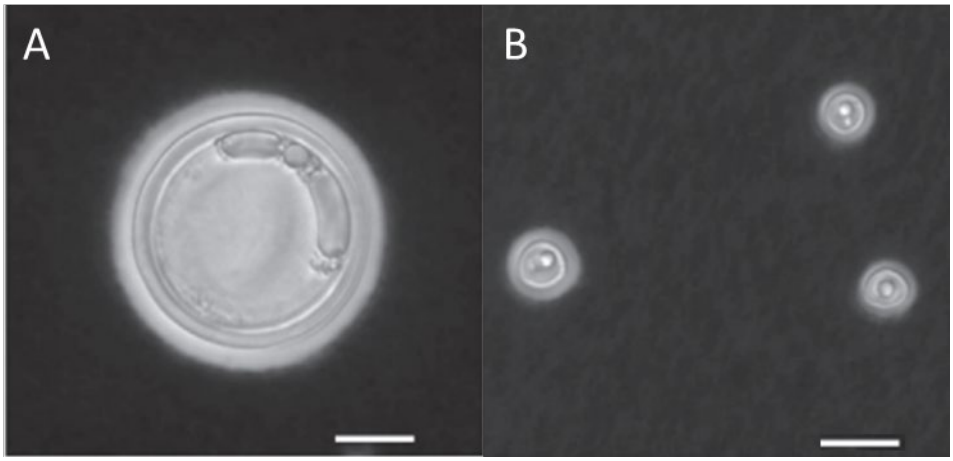

Figure 2.2: Differential interference contrast micrographs highlighting the size differences between a *Cryptococcus neoformans* titan cell (A) and cells of typical size (B). Scale bar = 10 µm. Figure modified from Mukaremera et al., 2018.

Qsp1 was previously shown to stimulate cell division and replication (Lee et al., 2007; Homer et al., 2016); therefore, more cells would be budding in the presence of this peptide. However, titan cells were found to form during the stationary growth phase, when cells are not actively budding (Trevijano-Contador et al., 2018). This may explain why fewer titans are produced in the presence of Qsp1. In contradiction, when the qsp1Δ mutant is grown to saturation at high cell densities (in the absence of Qsp1), it exhibited reduced numbers of titan cells similar in quantity to that of the wild type strain. This suggests that Qsp1 may not be the exclusive regulator of titan cell formation and that factors influencing titanisation are also determined by the growth conditions.

2.5.4 The mode of *Cryptococcus* quorum sensing mimics that of Gram-positive bacteria

Bacterial and fungal members both perform QS using small diffusible molecules that can accumulate in the extracellular environment and these molecules have diverse structures (Hogan, 2006). Interestingly, the QSMs isolated and characterised for fungi other than *C. neoformans* to date have all been organic, non-peptide compounds (such as tyrosol, farnesol and isoamyl alcohol) or growth factors (hydroxamic acid). This makes *C. neoformans* the only fungal organism currently known to use a peptide-based QS system, imitating that of Gram-positive bacteria (Lee et al., 2007). The Gram-positive bacterium, *S. pneumoniae*, produces the QS-regulating competence-stimulating peptide 1 (CSP1) which is 17 amino acids in length and is initially biosynthesised as a 41 amino acid long precursor (Håvarstein et al., 1995). Although there is no resemblance in peptide sequence between CSP1 and *Cryptococcus* Qsp1, it is apparent that there are similarities in size and processing between these peptides. Qsp1 is first produced as a 45 amino acid long propeptide that gets processed into the functional 11-mer QSM (Fig. 2.1 & Fig. 2.3). Moreover, the mode of QS peptide signal detection and transduction is similar between certain Gram-positive bacteria and *C. neoformans* in that the mature form of the QS peptides are imported via cell membrane-bound

transporters and directly interact with various response regulators (Homer et al., 2016; Debunne et al., 2017). It is hypothesised that *C. neoformans* developed this typically bacterial mode of peptide communication via convergent evolution since the apparatus involved lack a proximal bacterial ancestor (Homer et al., 2016).

2.5.5 Other cryptococcal quorum sensing molecules

Most bacterial or fungal organisms known to be capable of QS have at least two or more autoinducing molecules (Lazazzera, 2000; Abisado et al., 2018); it is, therefore, likely that *C. neoformans* shares this characteristic and other potential QSMs for this organism have not yet been identified or fully characterised. Before Qsp1 was directly implicated in QS, it was shown that pantothenic acid (PA; or vitamin B_5) produced by *C. neoformans* might also act as a potential QSM (Albuquerque et al., 2014). Purified PA could also recover the growth defect experienced by tup1Δ mutants. However, this recovery was found to be significantly weaker than when the mutants were exposed to purified Qsp1 or crude *C. neoformans* culture supernatant within which both PA and Qsp1 were isolated. This could indicate that PA is not a strong QSM when acting on its own and that Qsp1 and other metabolites may be stronger controllers of QS in *Cryptococcus*. Additionally, using liquid chromatography-mass spectrophotometry, various derivatives of PA were identified in the culture supernatant of *C. neoformans*, indicating that PA may first be modified before it can exhibit a strong QS effect (Albuquerque et al., 2014). To date, no direct link between PA and Qsp1 activity has been made. Since both QSMs appear to affect similar processes in *C. neoformans*, it is likely that they interact or regulate similar genetic and physiological pathways in the pathogen. Furthermore, because PA exhibited a weaker effect on *C. neoformans*, it may potentially act as an inducer of Qsp1 production either by directly affecting the transcription of *QSP1* or through influencing the Qsp1-regulating transcription factors. However, these are all theories that still need further investigation.

Although Qsp1 was directly implicated in QS regulation in *C. neoformans*, a spliceosomal variant of the *QSP1* gene, identified as Qsp2, is a peptide that has been shown to have similar phenotype recovery activity to Qsp1 (Lee et al., 2007). Another spliceosomal variant, Qsp3, was also identified but this peptide did not appear to show the same function as the other peptides. All peptide variants share the same secretion signal and “NFGAPGG” motif with slight differences in the 3-4 terminal residues of the mature peptide region (Fig. 2.3). This hints that the current proposed model of QS in *C. neoformans* (Fig. 2.1) may be more complex and could additionally involve interaction with other Qsp signalling molecules. The effect of Qsp2 and Qsp3 beyond recovering the phenotype of the *C. neoformans* tup1Δ mutant has not been investigated. Despite all this information available on QS in *C. neoformans*, relatively few studies have investigated PA and the QS peptides in relation to QS or other physiological mechanisms. This means that QS in *Cryptococcus* remains mostly unexplored

and further investigation could provide meaningful insight into the regulation of virulence for this pathogen.

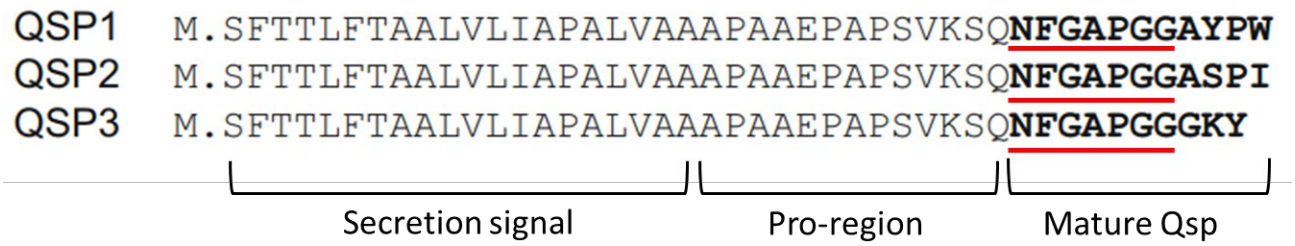

Figure 2.3: Comparison of the amino acid sequence of three quorum sensing-like peptides identified for *Cryptococcus neoformans*. The amino acid sequence shared between each mature peptide is underlined in red. Adapted from Kües and Navarro-González (2009).

2.6 The natural environment as a primer for the innate pathogenesis of *C. neoformans*

Obligate pathogens require growth within a host for survival, while facultative pathogens do not require this niche for growth and proliferation. Because *C. neoformans* is a facultative intracellular pathogen (Feldmesser et al., 2000), saprophytic (Staib, 1964) and usually found growing on decaying tree bark, pigeon excreta and in soil (Emmons, 1951; Emmons, 1955; Gibson and Johnston, 2015), this suggests that it does not naturally exist as a primary pathogen. Since cryptococcosis is a non-transmittable disease and infectious particles originate from the environment (Casadevall and Perfect, 1998), it is likely that the ecological stressors this pathogen face may have also selected for protective mechanisms that enable survival during host infections, only consequentially causing disease. Although many fungi are also exposed to similar stressors experienced by *C. neoformans* in the environment, they are not all capable of causing mammalian infections. These fungi may have adapted survival mechanisms dissimilar to that of *C. neoformans*, possibly due to unique stressors present in different niches within the same environment, which does not necessarily confer a selective advantage in mammalian hosts (Casadevall, 2005).

2.6.1 Interactions with amoebae

The ability of *C. neoformans* to survive within and escape macrophages during host immune responses has been attributed to its close association with predatory amoebae (Steenbergen et al., 2001). These amoebae are found in the same ecological niche as *Cryptococcus* spp. and feed on yeast by engulfing them via phagocytosis much in the same as macrophages. Once engulfed, yeast cells are enclosed in a vesicular sac called a food vacuole where they are digested (Geisen et al., 2016). In macrophages, infectious particles are fenced within phagolysosomes, where degradative enzymes and reactive electrophilic compounds are released to kill the infectious particles (Hünniger and Kurzai, 2018). It was found that amoebae-engulfed cells grew more rapidly and were capable of escaping via lytic exocytosis (Steenbergen et al., 2001), similar to what is observed after ingestion by macrophages (Diamond and Bennett, 1973; Lee et al., 1995). It is thus likely that *C. neoformans*

have adapted to overcome the attack by macrophages and persist in the human host through numerous encounters of amoeboid phagocytosis in the environment.

2.6.2 Stress-inducing abiotic factors in the ecological niche

2.6.2.1 High temperature and ultraviolet radiation

Besides attack by the immune system, *Cryptococcus* faces two other major stressors during host infection that it also encounters in its natural environment, namely, temperature elevation and nutrient limitation. Being mostly associated with the bark of trees, pigeon excreta and soil (Emmons, 1951; Emmons, 1955; Gibson and Johnston, 2015), *Cryptococcus* is exposed to high amounts of ultraviolet (UV) radiation and heat from the sun. Consequently, the yeast must also cope with elevated levels of oxidative stress. The cell wall of *C. neoformans* can contain a layer of melanin that is synthesised via a laccase enzyme (Rhodes et al., 1982) in the presence of a diverse range of phenolic and indolic compounds (Garcia-Rivera et al., 2005). In the environment, melanin substrates can be found in the form of tyrosine and a wide variety of by-products released by decaying wood matter. These by-products are also usually formed through the action of laccase enzymes from various saprophytic fungi that degrade wood as a carbon source (Kojima et al., 1990). Melanised cryptococcal cells have increased protection against UV radiation, oxidising agents and extreme drying (Cordero and Casadevall, 2017). This is due to the polymerous structure of melanin, which comprises a complex conjugated system containing many aromatic moieties. Energy transduction reactions can thus also be mediated by melanin, particularly from solar energy, to fuel the yeast's metabolic processes (Dadachova et al., 2007). Another characteristic feature of *Cryptococcus* cells that provide a similar protective feature against elevated temperature and oxidative stress is the presence of a thick polysaccharide capsule (Kozel et al., 1991; Zaragoza et al., 2008). During an attack by lytic enzymes and reactive oxygen species in the phagolysosomes of macrophages, both the polysaccharide capsule and melanin protect the yeast cells by acting as physical barriers and as electron scavengers (Liu et al., 1999; Zaragoza et al., 2008).

Moreover, mutants of *C. neoformans* incapable of producing melanin, or those with capsule defects, display reduced virulence in mouse models (Fromtling et al., 1982; Kwon-Chung et al., 1982). In the human host, small quantities of melanisable substrates, such as tyrosine derivatives, dopamine and L-dihydroxyphenylalanine (L-DOPA), can be found in the blood and cerebrospinal fluid (The Human Metabolome Database 4.0, http://www.hmdb.ca/). This suggests that *C. neoformans* could potentially use these substrates to synthesise melanin as a protective barrier against host-induced stress. Indeed, cryptococcal cells isolated from infected brain tissue appeared hyper-pigmented (Lee et al., 1996). Because *Cryptococcus* spp. need to survive temperature fluctuations and constant bombardment by UV radiation, these and other adapted mechanisms have allowed the yeast to also cope with elevated temperature and oxidative stress in the human host.

2.6.2.2 Nitrogen limitation

Nitrogen metabolism in fungi during low nitrogen availability

The natural, oligotrophic environment often contains a limiting and fluctuating supply of carbon and nitrogen available for fungi. These nutrients are essential energy sources required in high quantities by all living organisms (Ramachandra et al., 2014). To survive dynamic changes in nutrient availability, fungi have adapted flexible metabolic characteristics that aid in nutrient assimilation and have broadened their ability to use a variety of different nutrient sources. Ammonia, glutamine, glutamate and asparagine are the generally preferred nitrogen sources metabolised by fungi (Marzluf, 1997). In environments where these nitrogen sources are absent or in short supply, secondary sources of nitrogen may be used, such as proteins, free amino acids and polyamines.

Nitrogen metabolism in fungi is tightly regulated by nitrogen catabolite repression (NCR), a process that inhibits the uptake and catabolism of alternative nitrogen sources in the presence of preferred ones (Marzluf, 1997). Nitrogen metabolism in fungi is generally regulated by GATA factors (Wong et al., 2008), a class of transcription factors that share similar binding motifs between all fungi (Scazzocchio, 2000). In *C. neoformans*, nitrogen metabolism is chiefly regulated by the GATA-type transcription factor, Gat1 (Kmetzsch et al., 2011). When preferred nitrogen sources are depleted, Gat1 induces the expression of various NCR-repressed catabolic and transport genes that are necessary to acquire nitrogen from alternative supplies. In contrast, the regulation of nitrogen metabolism is much more complex in other yeast. For example, *C. albicans* employs two GATA-type transcription factors, Gat1 and Gln3 (Limjindaporn et al., 2003; Liao et al., 2008), to regulate nitrogen usage, while *S. cerevisiae* employs four (Gln3, Gat1, Dal80 and Gzf3) (Magasanik and Kaiser, 2002; Tudzynski, 2014).

During the early pulmonary infection of mammalian hosts, various pathogenic fungi induce the expression of genes necessary for amino acid biosynthesis, catabolism and transport (Hu et al., 2008; McDonagh et al., 2008). These genes are usually controlled at the transcriptional level by NCR (Wong et al., 2008), meaning that they would not have been expressed in the presence of favourable nitrogen sources (Marzluf, 1997). Preferred nitrogen sources are limiting in the human host, where available nitrogen is found primarily in the form of proteins (Ries et al., 2018). This suggests that nitrogen limitation (NL) is a key stressor faced by pathogenic fungi in the host environment. Therefore, acquired metabolic and physiological adaptations due to environmental stress may have also coincidentally allowed certain fungi to survive within mammalian hosts. However, due to differences in specific fungal niches, and thus variations in stressors encountered, not all survival mechanisms acquired by fungi also improve survival in mammalian hosts (Casadevall, 2005). The metabolic-induced changes by pathogenic fungi, particularly in response to NL, have been considered virulence factors because they serve the dual purpose of promoting fungal survival while also being necessary for pathogenesis during host infection (Ene et al., 2014).

Influence of nitrogen availability on the virulence composite of C. neoformans

In areas like the lungs, bloodstream and cerebrospinal fluid of humans, which *C. neoformans* is usually isolated from during disseminated infections (Maziarz and Perfect, 2016), available nitrogen is limiting. Here, metabolic adaptations acquired from environmental stress have allowed the pathogen to make use of unconventional nitrogenous compounds for survival. One example is the capability of *C. neoformans* to metabolise urea. Relative to other nitrogen sources found in pigeon excrement, one of the natural habitats of *C. neoformans* (Emmons, 1951; Emmons, 1955), uric acid contributes to approximately 70% of the total nitrogen (Hubálek, 1975). Also, uric acid and urea are present in the waste products released by animals into the soil (Barsoum and El-Khatib, 2017). The predominant forms of nitrogen in the blood and cerebrospinal fluid of the human body are free amino acids (glutamine, glutamate, asparagine and proline), allantoin, ammonium, urea and creatine (Lee et al., 2013a; The Human Metabolome Database 4.0, http://www.hmdb.ca/), with urea being the most abundant. *Cryptococcus* host enzymes capable of converting uric acid to allantoin and then to urea, which can be degraded further by urease enzymes into usable substrates for biosynthetic processes (Lee et al., 2013b). Ureases are important nitrogen-scavenging metalloenzymes biosynthesised by *C. neoformans*, enabling the yeast to produce ammonia and carbamate from urea as a nitrogenous substrate (Cox et al., 2000; Olszewski et al., 2004). Thus, during shortages in favourable nutrients in the environment, urea can serve as an important nitrogen source for *C. neoformans*. Mouse models simulating *C. neoformans* infection implicated the importance of ureases for virulence and invasion of the central nervous system (CNS), where urease deficient strains displayed reduced pathogenicity (Cox et al., 2000; Olszewski et al., 2004).

Previous studies have demonstrated that, in addition to increased urease production, the expression of other important virulence factors of *C. neoformans* is also modulated in response to local nitrogen concentrations. When grown in nutrient-limiting media with unfavourable nitrogen sources, such as urea, *Cryptococcus* displays a significant increase in polysaccharide capsule thickness and melanisation (Lee et al., 2011). Similar phenotypes were observed when various *C. neoformans* and *C. gattii* strains were cultured in media specifically limiting in nitrogen (Bosch et al., unpublished). In *C. neoformans*, these virulence factors are known to be controlled by nitrogen sensing pathways such as the target of rapamycin (TOR) and cyclic adenosine monophosphate-protein kinase A (cAMP-PKA) pathways, as well as their major downstream transcription factors (Cramer et al., 2006; Lee et al., 2011; Ries et al., 2018).

Although many other environmental factors may have influenced the development of pathogenesis, it appears that a combination of predation, temperature-related stress and NL are some of the key factors that have influenced the evolved pathogenesis of *C. neoformans*.

Fungal quorum sensing affected by nitrogen limitation

As previously discussed, physiological adaptations that have been necessitated in the ecological niche of *C. neoformans* have resulted in this organism developing mechanisms that make it an effective pathogen of mammalian hosts. Because NL has been shown to affect the virulence phenotype of *C. neoformans*, it is likely that QS responses, which are known to regulate virulence factor expression (Bandara et al., 2012), are also being influenced by this condition. For the ascomycete, *S. cerevisiae*, this has been confirmed (Chen and Fink, 2006). When grown in the presence of limiting nitrogen, and in response to high cell densities, *S. cerevisiae* was found to produce higher concentrations of QSMs and consequently showed a higher frequency of morphological switching from yeast to invasive filamentous growth. The observed phenotypic switching was attributed to the fact that the *S. cerevisiae* QSMs were found to upregulate the expression of *FLO11* (Chen and Fink, 2006), a gene encoding a glycosylphosphatidylinositol-anchored cell surface flocculin protein necessary for adhesion and invasive growth (Lo and Dranginis, 1998).

Overall, the role of QS in mediating nitrogen-limiting virulent responses for *C. neoformans* has not been investigated. Understanding the interplay between virulence factor expression, NL and QS may provide further insight into the physiology of *C. neoformans*, how it was primed for pathogenesis and identify novel solutions to treat cryptococcal infections.

2.7 Fungal drug resistance and treatment strategies

2.7.1 Acquired resistance to antifungal drugs

Many antifungal agents and antifungal treatment strategies involve targeting cellular components vital for the growth and survival of microorganisms. Targeting components with essential housekeeping functions results in a strong signal that forces cells to either adapt to this stress or die. The major drawback to these killing strategies is that they promote microbial evolution, which can lead to these organisms rapidly acquiring drug resistance (Cegelski et al., 2008; Fong et al., 2017). Long-term use of antimicrobial drugs, and their misuse, not only risks the chance of pathogens acquiring drug resistance but also reduces the numbers of naturally occurring microbes in the body. As a result, hosts could become more susceptible to infection as a loss in diversity of the human microbiome reduces competition for space and nutrients, which may likely facilitate the growth of invading, opportunistic species (Gilbert et al., 2014).

In terms of fungal infections, azoles and polyenes are the earliest and most commonly prescribed drugs for treatment (Chandrasekar, 2010). These drugs, which include fluconazole (FLC) and amphotericin B (AmB), respectively, disrupt cell membrane integrity and ergosterol (Holz, 1974; Bossche et al., 2009). This fungal sterol is required for maintaining cell membrane integrity and fluidity, regulating the movement of nutrients and waste across the cell membrane, as well as being

necessary for the proper functionality of membrane-bound enzymes (Parks and Casey, 1995). Due to the importance of ergosterol, the cell tries to cope with the stress that ergosterol-targeting drugs apply by either upregulating the production of this sterol (Bossche et al., 1992), overproducing drug efflux pumps to remove intracellular drugs (Sanglard et al., 1995) or mutate drug targets (Kelly et al., 1995; Kelly et al., 1999). This allows the cell to overcome the stress imposed by these antimycotic drugs and thus acquire resistance to them. A newer class of drugs prescribed for fungal infections are echinocandins. These drugs also target essential components of fungal cells (i.e. the cell wall) (Denning, 2002). Thus many cases of acquired resistance to echinocandins have also been reported (Hakki et al., 2006; Laverdière et al., 2006).

It is estimated that a global mortality of 10 million deaths will occur due to infectious diseases by the year 2050 if no new alternatives are developed to combat drug resistance in microbial pathogens (O'Neill, 2016). Currently, more than 300 million people worldwide are estimated to have acute fungal infections, of which 25 million risk dying from these infections (www.gaffi.org). In South Africa alone, over 4 million people contract fungal infections every year (Schwartz et al., 2019). This highlights the crucial need for innovative treatment solutions and approaches to antifungal drug design. However, developing antifungal drugs that do not also harm the host is challenging. This is because fungal cells and mammalian host cells are both eukaryotic and share similar genetic, metabolic and structural components (Mazu et al., 2016). Therefore, identifying distinct cellular components unique to fungi, which are then also targeted by an inhibitory drug, is difficult.

2.7.2 Current treatment measures against *C. neoformans* infections

Polyene-based treatment regimens have been shown to successfully reduce cryptococcal burden in the CNS and improve the survival of infected patients (Sloan and Parris, 2014). Because of the fungistatic nature of FLC, a successful outcome is poor and thus monotherapy with this drug is not recommended (Larsen et al., 1990; Schaars et al., 2006). The most effective clinical treatment regime for cryptococcosis, as recommended by the WHO, currently consists of AmB and flucytosine combination therapy. This treatment is done for two weeks; whereafter FLC is used to control and clear the infection (Smith et al., 2015). In cases where flucytosine is unavailable, patients are treated with a mixture of FLC and AmB (Perfect et al., 2010).

Despite AmB and flucytosine combination therapy being effective, as recently reported in a randomised trial (Molloy et al., 2018), long-term misuse of these antifungals has previously resulted in cases of *C. neoformans* acquiring resistance to them (Gago et al., 2016). Renal toxicity in patients is another issue faced when AmB is used (Perfect and Bicanic, 2015). Also, due to a lack of resources in developing regions, such as sub-Saharan Africa, AmB and flucytosine are not easily administered or available. Currently, Ghana and Rwanda are the only countries in sub-Saharan Africa with a registered and available supply of flucytosine (www.gaffi.org). This is concerning

because sub-Saharan Africa has the highest prevalence of individuals with HIV immune-suppression (Azevedo et al., 2016), which are consequently most susceptible to cryptococcal infections. Furthermore, the other major class of antifungal drugs available, echinocandins, are inactive against *C. neoformans* (May et al., 2016). In cognisance of the challenges imposed by current anticryptococcal treatment measures, more effective strategies need to be developed to combat *Cryptococcus* infections, especially using approaches that can feasibly be implemented in resource-poor countries.

2.8 Alternative strategies to combat drug resistance

In the past 20 years, there has been a drive to develop new antimicrobial treatment strategies in light of the constant battle with microbial drug resistance. Some of which include investigating alternative drug sources (Enioutina et al., 2017; Travis et al., 2018), repurposing already available drugs (Miró-Canturri et al., 2019), as well as using antipathogenic and antivirulence strategies (Fong et al., 2017). The two latter strategies, which involve targeting cascades of gene expression or virulence factors that are important for pathogenicity and not necessarily for survival, are theorised to stimulate a reduced selection pressure, thus making resistance development less likely (Cegelsky et al., 2008; Jakobsen et al., 2012; LaSarre and Federle, 2013; Kirienko et al., 2019). Generally, QS is known to control the expression of virulence factors (Bandara et al., 2012). Therefore, targeting components of the microbial QS response could affect pathogenesis and serves as a promising antipathogenic approach to new treatment solutions.

2.8.1 Quorum quenching

2.8.1.1 Inhibition of quorum sensing in pathogens

The interference or inhibition of microbial QS mechanisms (Dong et al., 2001), termed quorum quenching (QQ), is one example of an antipathogenic strategy. Inhibition of QS can be achieved either by direct interference with autoregulatory molecules or through inhibiting the binding and recognition of QSMs to the cell or genetic material. Various compounds have QQ properties against bacteria. These include small chemicals such as halogenated furanones and synthetic peptides that show structural similarity to natively produced bacterial QSMs (Lyon et al., 2000; Hentzer et al., 2003). Because these chemicals have little differences in structure when compared to their native counterparts, they mostly act by interfering with the recognition and transmission of QS signals by reducing free cellular QSM-binding receptors as they compete for these same binding sites (Lyon et al., 2000; Manefield et al., 2002). Other compounds include chemicals that inhibit key enzymes involved in QS, such as the histidine kinase sensor and enzymes involved in producing AHL intermediates (Hoang and Schweizer, 1999; Stephenson et al., 2000). It was previously shown that synthetic halogenated furanones and organosulfur compounds could inhibit QS-regulated processes in *P. aeruginosa* and that these compounds affected the expression of known genes implicated in QS (Hentzer et al., 2003; Jakobsen et al., 2012; Li et al., 2018; Li et al., 2019). As highlighted by

Azevedo et al. (2016), a few compounds have been shown to target key virulence factors of *C. neoformans*. However, no studies have reported on candidate compounds that affect QS directly for this pathogen.

2.8.1.2 Designing biological sensors using a quorum quenching approach

Various devices composed of biological entities, such as live cells or cellular components, have been designed to monitor and report relevant changes in systems (Su et al., 2011). Biosensors employing live cells usually consist of elements that sense a specific analyte and trigger an intracellular genetic response, such as the transcription and translation of a recombinant gene encoding a fluorescent protein that effectually provides a measurable signal (Adeniran et al., 2015). To investigate the potential of using a QQ approach when targeting microbial pathogens, biosensors have been designed to monitor the effect certain conditions or compounds have on the QS response of these pathogens. Recent advances in characterising QS in *C. neoformans* have revealed which genetic components play a crucial role in mediating this response (Homer et al., 2016). These genetic elements could be exploited to engineer a *C. neoformans* QS biosensor to monitor QS responses under different conditions, such as nutrient and temperature stress, as well as to screen a library of compounds with potential *Cryptococcus* QQ properties. The former approach has previously been demonstrated for the bacterial pathogen, *P. aeruginosa*. Zhang and co-workers (2018) developed a multicolour whole cell-based QS biosensor by fusing the promoters of four *P. aeruginosa* QS-responsive genes to different fluorescent reporter genes in a single plasmid construct. It was demonstrated that this recombinant bacterial pathogen could be used as a biosensor to simultaneously monitor the expression of the reporter genes from all four QS promoters in response to salt stress.

As little is known about the QS response of *C. neoformans*, a QS biosensor could serve as a valuable tool to further characterise this process under various conditions. One could even investigate whether other metabolites produced by *C. neoformans* impact the same processes as already known QSMs for this pathogen. This idea can be applied to other pathogenic fungi like *C. albicans* and could help progress antifungal drug research.

2.8.2 Garlic-derived organosulfur compounds as potential quorum sensing inhibitors

Garlic (*Allium sativum* L.) is a bulb crop commonly used as a food additive and renowned globally for its plethora of healing properties. One of the earliest records describing garlic as a medicine was in the *Codex Ebers Papyrus*, a document dating back to Ancient Egypt (1550 B.C.). Natural thiosulfinates derived from garlic cloves and garlic extracts, such as ajoene ((*E*/*Z*)-4,5,9-trithiadodeca-1,6,11-triene 9-oxide) and allicin (diallyl thiosulfinate), are responsible for most of the health benefits associated with the vegetable (Naznin et al., 2007). One garlic clove can release approximately 2.5 mg of allicin (Slusarenko et al., 2008), which is an unstable, thiol-reactive

organosulfur compound that readily decomposes to form diallyl disulfide (DADS) and diallyl trisulfide (DATS). Further degradation of allicin eventually produces the late, second-generation organosulfur by-product, ajoene. Naturally, garlic releases allicin as a defensive mechanism to deter organisms from ingesting them and gives the vegetable its distinctive smell (Gruhlke et al., 2019). Garlic extracts and a range of garlic-derived organosulfur compounds (Fig. 2.4) were shown to have antibacterial (Naganawa et al., 1996), antifungal (San-Blas et al., 1989; Davis et al., 2003), antiprotozoal (Urbina et al., 1993; Perez et al., 1994), antiviral (Tatarintsev et al., 1992; Weber et al., 1992), anticancer (Kaschula et al., 2016; Kaschula et al., 2019), antithrombic (Block and Ahmad, 1984) and quorum quenching (Bjarnsholt et al., 2005; Jakobsen et al., 2012) activities.

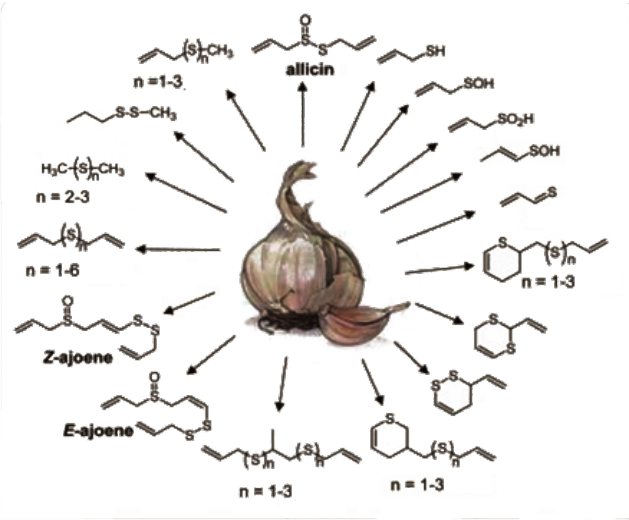

Figure 2.4: Range of potential organosulfur compounds present in garlic extracts. The main bioactive ingredients of garlic extracts, (E/Z)-ajoene and allicin, are highlighted with bold text. Figure adapted from Kaschula et al., 2010.

Previously, ajoene was shown to inhibit the expression of *P. aeruginosa* virulence genes which are regulated by QS (Jakobsen et al., 2012). The compound was further found to reduce the production of butanoyl homoserine lactone (C4-HSL), a *Pseudomonas* QSM, in a dose-dependent manner. Additionally, Fong et al. (2017) demonstrated that ajoene and other synthetic disulfide containing compounds could inhibit QS as well as the expression of the three *Pseudomonas* virulence factors: elastase, pyocyanin and rhamnolipid production. Conversely, they demonstrated that all tested synthetic analogues lacking disulfide functional groups failed to affect QS. Therefore, the inclusion of disulfide functional groups in drug design could be important for the discovery of novel QQ drugs.

Although many extracts and purified compounds derived from herbal sources still require strong scientific evidence to support their use in the clinical setting, garlic-derived organosulfur compounds appear promising, particularly for the treatment of cryptococcal infections. In resource-poor countries where the most effective anticryptococcal drugs are too expensive to administer or largely unavailable (Perfect and Bicanic, 2015), garlic is abundant and easily accessible (Diriba-Shiferaw,

2016). Compared to AmB, which requires expensive intravenous administration (Bindschadler and Bennet, 1969; Perfect and Bicanic, 2015), allicin is rapidly and almost completely absorbed when taken orally (Lin et al., 2015a). Another advantage to using ajoene, allicin and some of their synthetic derivatives is attributed to their lipid solubility. Most antimicrobial drugs are ineffective against infectious agents that manage to survive the host's immune response and cross the blood-brain barrier. This is because the majority of drugs are unable to penetrate tissues of the brain. However, due to its highly lipid-soluble nature, allicin can cross tight junctions protecting the blood vessels connected to the brain (Miron et al., 2000). Therefore, allicin and synthetic derivatives thereof could theoretically still target *Cryptococcus* cells in the CNS of patients already presenting symptoms of cryptococcal meningoencephalitis. Previous studies have shown that allicin and raw garlic extracts can inhibit the growth of *C. neoformans* (Yamada and Azuma, 1977; Davis et al., 1990; Khan and Katiyar, 2000; Rehman and Mairaj, 2013). Although these naturally-derived compounds would likely not replace current antifungal drugs used to treat cryptococcosis, they could be used in combination therapy with FLC to improve fungal clearance in cases where AmB cannot be administered, or flucytosine is unavailable.

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CHAPTER 3

RESEARCH RESULTS I

Characterising the link between nitrogen limitation, quorum sensing and virulence in *Cryptococcus neoformans*

Chapter 3: Characterising the link between nitrogen limitation, quorum sensing and virulence in *Cryptococcus neoformans*

3.1 Abstract

In its natural environment, *Cryptococcus neoformans* experiences many abiotic stressors. Some of which include predation, fluctuating temperatures and nitrogen limitation (NL). In response to limiting concentrations of non-preferred nitrogen sources, *C. neoformans* induces the expression of important virulence factors, such as the polysaccharide capsule, ureases and melanin. Generally, quorum sensing (QS) is known to regulate virulence factor production in many pathogenic microorganisms. Recently, a small QS peptide (Qsp1) was found to regulate QS and virulence factor expression in *C. neoformans*. The effect NL has on cryptococcal virulence factors and their regulation by QS under these conditions has not been explicitly characterised. In this study, nitrogen-limiting media (NLM) containing 0.5 g/L NH_4Cl (NLM-0.5) or 0.2 g/L NH_4Cl (NLM-0.2) as the sole nitrogen source were used to assess virulence factor expression. It was found that decreasing nitrogen concentrations resulted in an increase in capsule thickness, urease activity and melanisation. To characterise whether QS controls the observed virulence phenotype under NL, we compared the virulence factor phenotype of *C. neoformans* H99 and a *C. neoformans* CK0289 QS mutant. Preliminary evidence suggested that Qsp1 plays a role in regulating capsule enlargement, urease activity and melanisation under conditions of NL. Exposing the QS mutant to synthetic mature Qsp1 (mQsp1) recovered the capsule phenotype to that of the wild type strain. Exposure to mQsp1 did not affect melanisation but variably affected urease activity. Moreover, mQsp1 appears necessary to induce ergosterol biosynthesis under NL and elevated temperature. Therefore, NL does affect cryptococcal virulence factor expression and may be regulated by a Qsp1-mediated QS response.

3.2 Introduction

The pathogenic basidiomycete, *Cryptococcus neoformans*, contains four major stress-activated signalling pathways that detect and respond to an assortment of environmental stressors, and are also important for virulence. These signalling pathways include the high-osmolarity glycerol (HOG), Ca^{2+}/calcineurin, Ras and protein kinase C/Mpk1 mitogen-activated protein kinase (MAPK) signalling pathways (Jung and Bahn, 2009). In particular, the HOG pathway regulates ergosterol biosynthesis as well as the expression of virulence genes responsible for capsule formation and melanisation in *C. neoformans* (Ko et al., 2009). An environmental factor that strongly influences stress-activated signalling pathways is nutrient availability, particularly low carbon supply and nitrogen limitation (NL) (Xue et al., 2004). In fungi, genes involved in nitrogen metabolism are transcriptionally controlled by nitrogen catabolite repression (NCR), a process that prevents the metabolism of alternative nitrogen sources when preferred nitrogenous compounds are available (Marzluf, 1997). A class of nitrogen sensing transcription factors, called GATA factors, are primarily responsible for the regulation of

NCR (Wong et al., 2008). In *C. neoformans*, when preferred nitrogen sources are limiting, the GATA-type transcription factor, Gat1, induces the expression of genes previously inhibited by NCR (Kmetzsch et al., 2011). These genes are generally responsible for the catabolism and transport of alternative nitrogen sources. Furthermore, Lee and co-workers (2011) demonstrated that in addition to upregulated urease activity, C*. neoformans* displays pronounced melanisation and polysaccharide capsule phenotypes in minimal media containing non-preferred nitrogen sources. Similarly, using ecologically relevant nitrogen concentrations determined by Vreulink et al. (unpublished data), a selection of *C. neoformans* and *C. gattii* strains were found to produce thicker capsules and more melanin during NL (Bosch et al., unpublished).

Saccharomyces cerevisiae, an ascomycetous yeast, undergoes a morphological transition to pseudohyphal growth in response to NL (Gimeno et al., 1992). Genes involved in the induction of filamentous growth are activated via components of the MAPK and protein kinase A pathways (Liu et al., 1993), which are both sensors of NL and function independently of one another (Rupp et al., 1999). Furthermore, morphological switching was found to be regulated by quorum sensing (QS) – *S. cerevisiae* secreted higher concentrations of aromatic alcohols previously reported as QS molecules (QSMs) during NL, which enhanced filamentation (Chen and Fink, 2006). Pseudohyphal growth can confer a selective advantage in the natural habitat, enabling microorganisms to avoid an unfavourable environment and to forage for nutrients that are in short supply (Gancendo, 2001). Consequently, for certain pathogenic fungi, morphological switching permits the invasion of host tissue and virulence (Li and Nielsen, 2017). Taken together, these findings suggest a possible relationship between the three factors: NL, QS and virulence. Since NL appears to have a strong influence on the virulence phenotype of *C. neoformans*, it is possible that QS, a process generally regulating virulence in most microorganisms (Bandara et al., 2012), may also be affected. However, the interplay between NL and QS, and how this affects virulence factor expression and the physiological responses of *C. neoformans* is not well characterised.

An 11-mer peptide (Qsp1) was previously identified as an important QSM for *C. neoformans* (Lee et al., 2007; Homer et al., 2016). The peptide is produced in a cell density-dependent manner and regulates the expression of specific transcription factors that act as global regulators of virulence-associated genes. A *C. neoformans* qsp1Δ mutant unable to produce Qsp1 showed reduced mortality of infected mice and displayed variability in capsule thickness and melanisation when compared to a wild type *QSP1* strain (Homer et al., 2016). The polysaccharide capsule of *C. neoformans* is a well-studied outer cell wall polymer that facilitates survival in the natural environment as well as during mammalian host infection (Fromtling et al., 1982; Wu et al., 2008; Zaragoza et al., 2008). The capsule serves as an important virulence factor and is often more prominently expressed during infection in host lung tissue, blood and cerebrospinal fluid (Feldmesser et al., 2001), where available nutrients, particularly nitrogen, is in short supply (Lee et al., 2013).

Laccase, a phenoloxidase enzyme, catalyses the cascade responsible for the conversion of several phenolic and indolic substrates to melanin (Garcia-Rivera et al., 2005). In the environment, *C. neoformans* has been isolated from wood and the decaying cavities of trees (Lazèra et al., 1996; Randhawa et al., 2001), which harbours many potential substrates for laccase enzymes (Kojima et al., 1990). Both the polysaccharide capsule and melanin protect cells from ultraviolet (UV) radiation, oxidative stress and predation by amoebae (Steenbergen et al., 2001; Cordero and Casadevall, 2017). Similarly, during host infection, these virulence factors provide protection against attack by macrophages, the killing effect of reactive electrophilic species as well as from host physiological temperature (Liu et al., 1999; Zaragoza et al., 2008). Since NL influences the QS response of yeast, and QS generally regulates virulence factor expression (Bandara et al., 2012), it is possible that the previously observed effects of NL on the virulence phenotype of *C. neoformans* may also be modulated by changes in its QS response.

In this study, we aimed to investigate the potential link between NL, QS and the virulence phenotype of *C. neoformans*. This was done by measuring phenotypic differences in the polysaccharide capsule size, melanisation and urease activity between *C. neoformans* H99 and a *C. neoformans* CK0289 QS mutant during NL.

3.3 Materials and methods

All reagents were supplied by Merck (Darmstadt, Germany) unless otherwise indicated.

3.3.1 Yeast strains

The *C. neoformans var. grubii* H99 (serotype A) was kindly provided by Dr. Joseph Heitman (Duke University, Durham, NC), and the *C. neoformans* CK0289 QS mutant (qsp1Δ; H99 background) strain was a kind gift from Prof. Hiten Madhani (University of California, San Francisco, CA) (Homer et al., 2016).

3.3.2 Media and reagents

Yeast extract-peptone-dextrose (YPD) medium was prepared with 20 g/L peptone, 10 g/L yeast extract and 20 g/L glucose. Solid media contained 1.5% (w/v) agar. Two nitrogen-limiting media (NLM) were prepared using 11.7 g/L Yeast Carbon Base (BD Life Sciences, New Jersey, USA) supplemented with either 0.5 g/L ammonium chloride (NH_4Cl; NLM-0.5) or 0.2 g/L NH_4Cl (NLM-0.2) as the sole nitrogen source. The media were adjusted to pH 5.5 with NaOH.

3.3.3 Virulence phenotype comparisons between *C. neoformans* H99 and *C. neoformans* CK0289

3.3.3.1 Preparation of yeast inocula

Yeast cultures were prepared in test tubes by inoculating a single yeast colony into 5 mL YPD broth and incubated for 24 h at 30°C on a rotating wheel. Cells were harvested, washed twice and resuspended in sterile phosphate-buffered saline (PBS) solution before transfer to test medium at specified cell densities. PBS was used for all wash steps, and harvesting was performed using centrifugation (5000 x *g*, 1 min) unless stated otherwise.

3.3.3.2 Polysaccharide capsule thickness measurements

Washed yeast cells were inoculated to a cell density of 1 x 10^6 cells/mL in 5 mL NLM-0.5 or NLM-0.2 and grown to saturation for 48 h at 30°C or 37°C on a rotating wheel. Following incubation, the cells were harvested, washed twice and stained with 10% (m/v) nigrosin before capturing images of the encapsulated cells with a Nikon Eclipse E400 fluorescence microscope (Laboratory Optical Service, Inc., Virginia, USA). Yeast cell radius and total radius including the capsules were measured for 80-100 cells per treatment using ImageJ 1.51 j8 software (National Institutes of Health, Bethesda, USA) (Frases et al., 2009). Capsule thickness was determined by subtracting the yeast cell radius from the total radius. All treatments were performed in triplicate with data normalised against mean cell diameter.

3.3.3.3 Laccase activity

Laccase activity was tested using an approach described by Eisenman et al. (2007) with modifications. Cells from overnight cultures were harvested, washed twice and inoculated to a cell density of 1 x 10^6 cells/mL into test tubes containing 5 mL NLM-0.5 or NLM-0.2. Inoculated cells were incubated to saturation for 48 h at 30°C or 37°C on a rotating wheel. Thereafter, cultures were harvested, washed twice and inoculated to a cell concentration of 5 x 10^6 cells/mL in test tubes containing 5 mL reverse osmosis-purified (RO) water with 5 mM 3,4-dihydroxy-L-phenylalanine (L-DOPA, TLC grade). Samples were incubated at 30°C or 37°C in the dark on a rotating wheel. Absorbance of each sample was measured after 24 h at a wavelength of 490 nm. All treatments were performed in triplicate and a control included cells without L-DOPA.

3.3.3.4 Urease activity

Assays were performed in the same manner as described for laccase activity. After saturated growth in NLM-0.5 or NLM-0.2, cells were harvested, washed twice with RO water and resuspended to a cell concentration of 5 x 10^6 cells/mL in test tubes containing 5 mL assay medium. The assay medium contained 0.66 mM urea as a substrate and 0.012 g/L phenol red pH indicator. Samples were incubated at 30°C or 37°C on a rotating wheel. An absorbance reading of each sample was taken

after 24 h at a wavelength of 570 nm. The experiment was performed in triplicate, and a control containing only cells and indicator (no substrate) was included.

3.3.4 Virulence phenotype recovery assays

Virulence phenotype recovery was tested by exposing the *C. neoformans* qsp1Δ mutant strain to synthetic mature Qsp1 (mQsp1) during all abovementioned virulence factor assays (section 3.3.3). The QS peptide was synthesised via GL Biochem (Shanghai) Ltd. (Shanghai, China). Slight modifications to the polysaccharide capsule, laccase and urease assays were made. Briefly, cultures of the *C. neoformans* qsp1Δ mutant strain were washed and resuspended to a cell density of 1 x 10^6 cells/mL in test tubes containing 1 mL NLM-0.5 or NLM-0.2. Only the nutrient and temperature conditions inducing the strongest virulence phenotype in *C. neoformans* H99 were used for the phenotype recovery assays. For the laccase and capsule assays specifically, cells were incubated for 48 h at 37°C in NLM-0.2. However, for the urease assay, cells were incubated for 48 h at 30°C in both NLM-0.5 and NLM-0.2. To recover the virulence phenotype, mQsp1 was added at a final concentration of 80 µM to each treatment throughout the entire incubation period (48 h) or only during the final 24 h of incubation (Fig. 3.1). For measuring the thickness of the polysaccharide capsule, cells were harvested after incubation and viewed using the same method described previously. For the laccase and urease assays, cells were harvested after incubation, washed twice and resuspended to a cell density of 5 x 10^6 cells/mL in 1 mL of the respective test media. The cells were incubated for a further 24 h at either 30°C (urease) or 37°C (laccase) on a rotating wheel, whereafter absorbance readings were taken as described previously. The wild type *C. neoformans* H99 strain was included as a positive control. All treatments were performed in triplicate and controls without peptide exposure or substrate were also included.

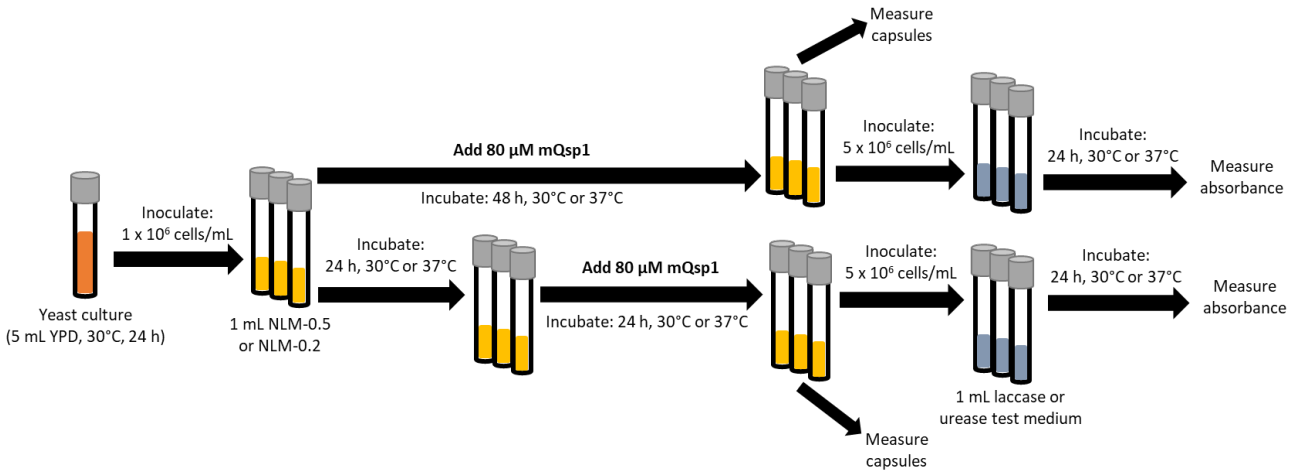

Figure 3.1: Schematic diagram of the virulence phenotype recovery assay (section 3.3.4), highlighting the different periods of exposure to synthetic mature Qsp1 (mQsp1).

3.3.5 Ergosterol quantitation

The method of fungal ergosterol quantitation, as described by Samie et al. (2019) was performed with modifications. These included inoculating cells into 250 mL conical flasks containing 25 mL NLM-0.5 or NLM-0.2. For each treatment, two separate flasks with 25 mL media were inoculated using the same colony suspension (after harvesting, these were combined to obtain a final culture volume of 50 mL). Flasks were incubated at 30°C or 37°C for 48 h on an orbital shaker (120 rpm) followed by harvesting (centrifugation at 2800 x *g* for 6 min) and washing with sterile RO water. Wet pellet weight measurements were taken as described previously. Each treatment was performed in triplicate.

3.3.6 Data visualisation and statistical analyses

All box and scatter plots were generated using an online software tool available at https://gabrifc.shinyapps.io/raincloudplots/ (Allen et al., 2019). Mean values were plotted with error bars denoting the standard error of the mean. For all datasets, the analysis of variance (ANOVA) was measured using one-way ANOVA, where the mean values were compared using Tukey's honestly significant difference post hoc test for multiple comparisons with a significance level of 0.05. Statistical analyses were performed using Statistica (version 13, TIBCO Software Inc., USA).

3.4 Results and discussion

Growth media containing nitrogen concentrations that *C. neoformans* encounters in its natural environment were used in this study in order to simulate the nitrogen-limiting-induced stress likely experienced by the pathogen. NLM-0.5 contains a total nitrogen concentration of 0.5 g/L – an approximate ammonia concentration previously quantified from the woody substrate of oak trees in Stellenbosch Central (South Africa) that *C. neoformans* strains have been isolated from (Vreulink et al., unpublished). The nitrogen concentration used in NLM-0.2 (0.2 g/L) represents more than half of the nitrogen concentration in NLM-0.5. Because this study solely focused on characterising cryptococcal behaviour in the presence of ecologically relevant nitrogen concentrations, no comparisons were made using treatments with nitrogen abundance or the limitation of other nutrient sources such as carbon.

3.4.1 Nitrogen limitation increases capsule thickness and is mediated by Qsp1

Changes in the capsule thickness of *C. neoformans* H99 was measured during growth during NL and compared to that of the *C. neoformans* CK0289 QS mutant. A trend of increased capsule thickness with reduced nitrogen concentrations was observed for both *C. neoformans* strains tested. At 30°C (Fig. 3.2A), *C. neoformans* H99 displayed significantly thicker capsules (14% increase) when grown in NLM-0.2 (0.293±0.003 µm) compared to growth in the higher nitrogen content medium, NLM-0.5 (0.258±0.004 µm) ($P < 0.001$). Our data confirmed previous observations made for *C. neoformans* H99 grown under the same conditions as well as other *C. neoformans* and

C. gattii strains (Bosch et al., unpublished). Similarly, *C. neoformans* CK0289 had a significantly thicker capsule (19% increase) after growth in NLM-0.2 (0.156±0.002 µm) compared to growth in NLM-0.5 (0.131±0.002 µm) ($P < 0.001$) at 30°C. However, the average capsule thickness of *C. neoformans* CK0289 under both NL conditions was significantly reduced (49% and 47% in NLM-0.5 and NLM-0.2, respectively) compared to the capsule of *C. neoformans* H99 at 30°C ($P < 0.001$).

Comparing the capsule thickness of cells during all treatments, thicker capsules were evident at 37°C. Relative to 30°C, *C. neoformans* H99 and *C. neoformans* CK0289 cells displayed a 0.4% and 14% increase in capsule thickness in NLM-0.5, respectively, and a 9% and 78% increase in NLM-0.2, respectively, at 37°C (Fig. 3.2). At 37°C, the average capsule thickness of *C. neoformans* CK0289 under both NL conditions was significantly reduced (74% in NLM-0.5 and 16% in NLM-0.2) compared to the capsules of *C. neoformans* H99 (Fig. 3.2B; $P < 0.001$). This suggests that QS may play a role in modulating capsule thickness during NL and high-temperature stress.

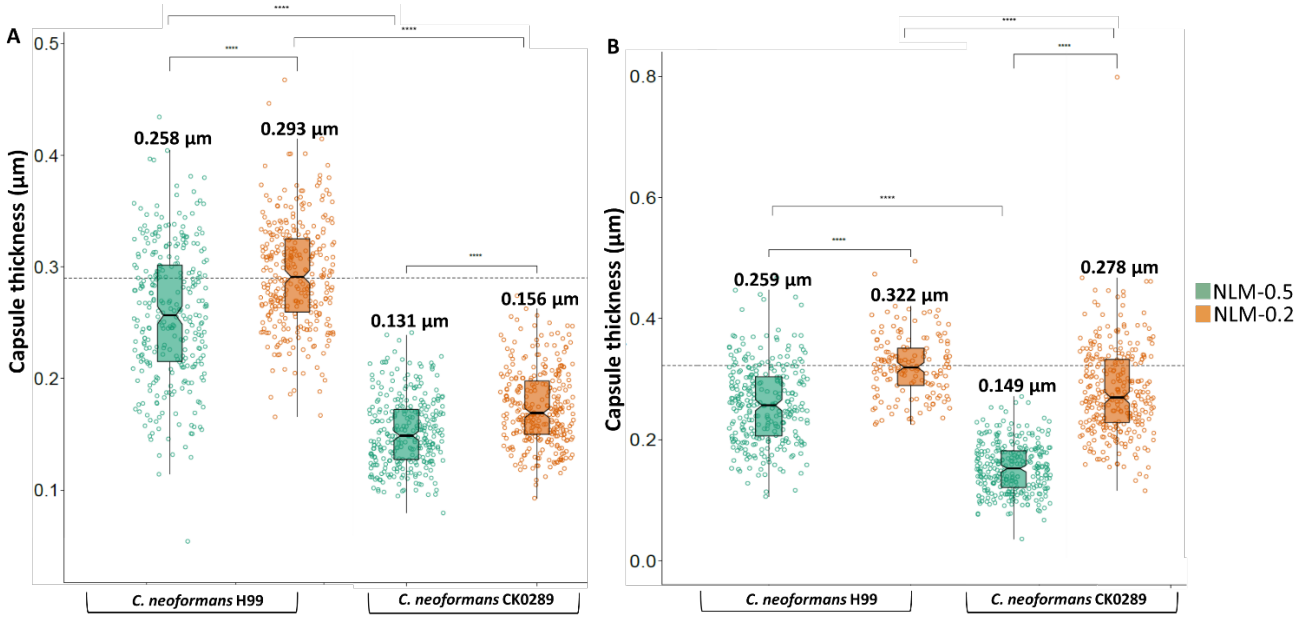

Figure 3.2: Capsule thickness of *Cryptococcus neoformans* strains grown under two nitrogen-limiting conditions at 30°C (A) and 37°C (B). The average capsule thickness for each condition is indicated above each boxplot. The dashed line in each graph indicates the average capsule thickness for *C. neoformans* H99 when grown in nitrogen-limiting media with 0.2 g/L NH_4Cl (NLM-0.2).

In its primary ecological niche, such as the decaying matter of trees, *C. neoformans* is most likely constantly under stress, including NL and temperature stress (Lee et al., 2013). Similarly, *C. neoformans* experiences these stresses during mammalian host infection, where the yeast often presents thick capsules (Rivera et al., 1998). The yeast has likely evolved polysaccharide production as a stress response in order to cope with the threat of desiccation, due to high temperature, and UV radiation in the environment (Wu et al., 2008; Zaragoza et al., 2008), consequently also providing protection in the environment of the infected host. Although the exact reason for thicker capsules in

response to low nitrogen is unclear, molecular evidence supporting the role of nitrogen availability in the pathogenicity of *C. neoformans* has previously been described. The NCR pathway, a global regulatory circuit, found in ascomycetous yeasts is also present in *C. neoformans*. Gat1/Are1-mediated inactivation of NCR, in the absence of preferred nitrogen sources, not only activates genes for nitrogen scavenging but also controls the coordinated production of other virulence factors such as the capsule and melanisation (Kmetzsch et al., 2011; Lee et al., 2011).

Vij et al. (2018) previously demonstrated that an increase in capsule size increases the buoyancy of *C. neoformans*, which helps disperse the non-motile cells in an aqueous environment. During host infections, increased buoyancy could help with the dissemination of the yeast from the lungs to the brain via the bloodstream. Therefore, in an environmental or clinical setting, when *C. neoformans* is starved of nitrogen (or other nutrients) in its immediate vicinity, it may produce a thick capsule to assist with movement to another location with available nutrients. A more probable explanation is the role of NL during phagocytosis by host immune effector cells, such as macrophages. After ingestion by macrophages, *C. neoformans* experiences a nutrient-poor environment whereafter the cells are bombarded with a cocktail of reactive electrophilic species and lytic enzymes (Hünniger and Kurzai, 2018). Because the polysaccharide capsule protects the cells against the killing factors produced by macrophages during host infection (Zaragoza et al., 2008) and predation by amoebae in the environment (Steenbergen et al., 2001), NL could, therefore, serve as a signal for *C. neoformans* to increase its capsule size as a protective stress response to promote survival. Moreover, capsule enlargement may be a general stress response induced by the cells as capsule thickness was found to be affected by other factors such as osmotic pressure (Dykstra et al., 1977), carbon dioxide levels (Granger et al., 1985) and iron limitation (Vartivarian et al., 1993).

Overall, *C. neoformans* H99 produced the thickest capsules when grown in NLM with the lowest nitrogen concentration (NLM-0.2) at both 30°C and 37°C, supporting the role of nitrogen in modulating the expression of this virulence factor. The *C. neoformans* H99 strain had thicker capsules than the *C. neoformans* CK0289 QS mutant during all treatments, which suggests that Qsp1 also plays a role in capsule thickness. Taken together, this suggests that there is a link between NL and QS and that these factors also influence cryptococcal virulence.

Phenotype recovery experiments with mQsp1 provided further evidence for the link between QS and NL. The *C. neoformans* CK0289 strain was grown with or without the presence of mQsp1 in NLM. Theoretically, mQsp1 exposure should recover the QS mutant's virulence phenotype and result in it producing capsules with similar thickness to that of *C. neoformans* H99. A significant increase in the capsule thickness of *C. neoformans* CK0289 treated with mQsp1 was observed when compared to the untreated cells (Fig. 3.3) ($P < 0.001$). An exposure period of 24 h to 80 µM mQsp1 was sufficient to recover *C. neoformans* CK0289's capsule phenotype from 0.154±0.003 µm to 0.272±0.005 µm

(77% increase), similar to the capsule thickness of the *C. neoformans* H99 (0.273±0.004 µm). Prolonged exposure (48 h) resulted in a further 18% increase in the *C. neoformans* CK0289 mutant's capsule (0.332±0.006 µm) compared to the 24 h treatment, producing capsules thicker than even *C. neoformans* H99. These results confirmed that QS is necessary for the expression of a thicker polysaccharide capsule during NL at 37°C. Because a threshold concentration of Qsp1 needed to induce a QS response in *C. neoformans* has not been defined in the literature, a concentration of 80 µM may be much higher than what would naturally be produced by *C. neoformans*. This may be the reason why prolonged exposure to the peptide resulted in *C. neoformans* CK0289 producing thicker capsules than the H99 strain.

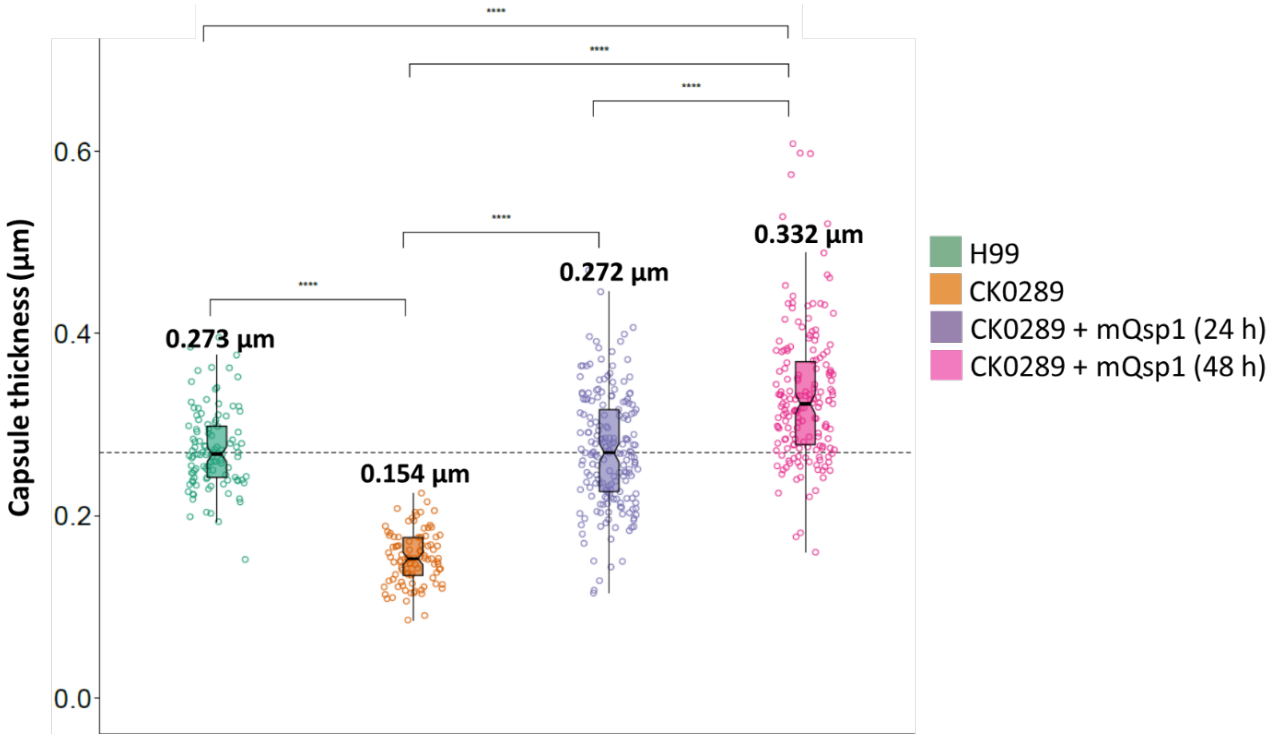

Figure 3.3: Capsule thickness of *Cryptococcus neoformans* strains grown at 37°C with or without exposure to synthetic mature Qsp1 (mQsp1) in nitrogen-limiting media containing 0.2 g/L NH_4Cl (NLM-0.2). The quorum sensing mutant strain, *C. neoformans* CK0289 (qsp1Δ), was exposed to mQsp1 for either 24 h or 48 h, as indicated. The average capsule thickness for each condition is indicated above each boxplot. The dashed line indicates the average capsule thickness of the unexposed *C. neoformans* H99 strain (0.273±0.004 µm).

When performing the capsule phenotype recovery assay with 33.3 µM mQsp1, no change in the thickness of the CK0289 QS mutant's capsule was detected (data not shown). This was also the case when tested for the recovery of laccase and urease activity (section 3.4.2.; data not shown). The synthetic Qsp1 was demonstrated to be functional at a concentration of 33.3 µM when recovering the *C. neoformans* CK0289 mutant's colony morphology phenotype on YPD medium (see Chapter 5, section 5.4.1.3). Previously, concentrations ranging from 3.3 nM to 33.3 µM mQsp1 were shown to functionally recover the growth defect of a *C. neoformans* tup1Δ (serotype D) mutant grown on YPD medium (Lee et al., 2007). Homer et al. (2016), however, showed that a concentration as

low as 1 μM in 10% (v/v) Sabouraud's medium could recover the secreted endoprotease profile and capsule phenotype of the qsp1Δ mutant. This variability implies that cryptococcal QS responses are not straightforward and are strongly affected by growth conditions. The differences in growth media, culture cell density and exposure time of mQsp1 may affect the measured phenotypes. Nevertheless, NL was demonstrated to modulate capsule enlargement and that this was in part controlled by a Qsp1-regulated response.

3.4.2 Expression of laccase and urease enzymes vary under nitrogen limitation and is likely not regulated by Qsp1

Changes in cryptococcal melanisation in response to NL was determined by measuring the activity of the copper enzyme, laccase, which converts exogenous diphenolic and indolic substrates such as L-DOPA to melanin (Rhodes et al., 1982; Garcia-Rivera et al., 2005). No laccase activity was detected at 30°C for *C. neoformans* H99 (Fig. 3.4). However, there was a 34X increase in the absorbance of melanin intermediates when grown in NLM-0.2 (0.102±0.001) compared to NLM-0.5 (0.003±0.001) at 37°C ($P < 0.001$). In contrast, relatively no laccase activity was detected under all nitrogen and temperature conditions tested for the *C. neoformans* CK0289 QS mutant. This suggests that Qsp1 may be essential for melanin production when *C. neoformans* experiences both NL and temperature stress. Previously, Chaskes and Tyndall (1975) demonstrated that *C. neoformans* only produced the melanin pigment between 2-7 days when grown in L-DOPA-supplemented media containing ammonium sulfate as the nitrogen source. Using other less preferred nitrogen sources, a minimum of 48 h was required before pigment was detected. Although the authors used different nitrogen sources and solid media, their observations may explain why relatively little melanisation occurred in this study as the yeast cells were only exposed to L-DOPA for 24 h.

To determine whether the increase in melanisation of the wild type strain during NL and the elevated temperature was attributed to QS, the *C. neoformans* CK0289 was exposed to 80 μM mQsp1. No recovery of laccase activity was evident after 24 h exposure to the QS peptide (Fig. 3.5). Although a slight 1.8X increase in melanisation was observed after 48 h of exposure (0.018±0.003) relative to the unexposed control (0.010±0.001), this increase was not statistically significant ($P = 0.9$) and was 5.7X lower than the absorbance measured for the wild type strain (0.102±0.001). Overall, these results imply that under NL and temperature stress, QS via Qsp1 may not affect the expression of cryptococcal laccase enzymes and melanisation.

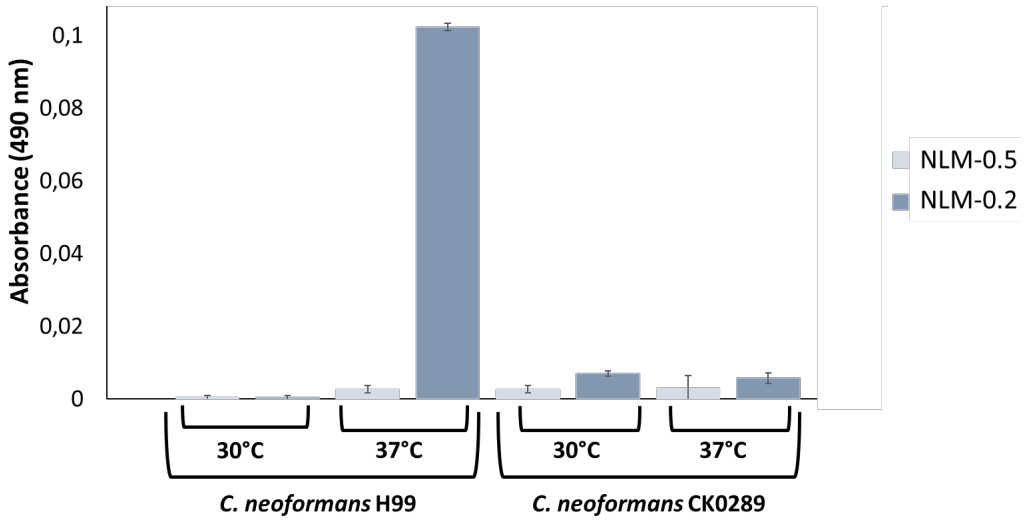

Figure 3.4: Melanisation of *Cryptococcus neoformans* strains grown in nitrogen-limiting media with either 0.5 g/L NH_4Cl (NLM-0.5) or 0.2 g/L NH_4Cl (NLM-0.2) at different temperatures. Melanisation was recorded as the absorbance of melanin intermediates produced via laccase activity at a wavelength of 490 nm. Error bars represent standard error of the mean (n = 3).

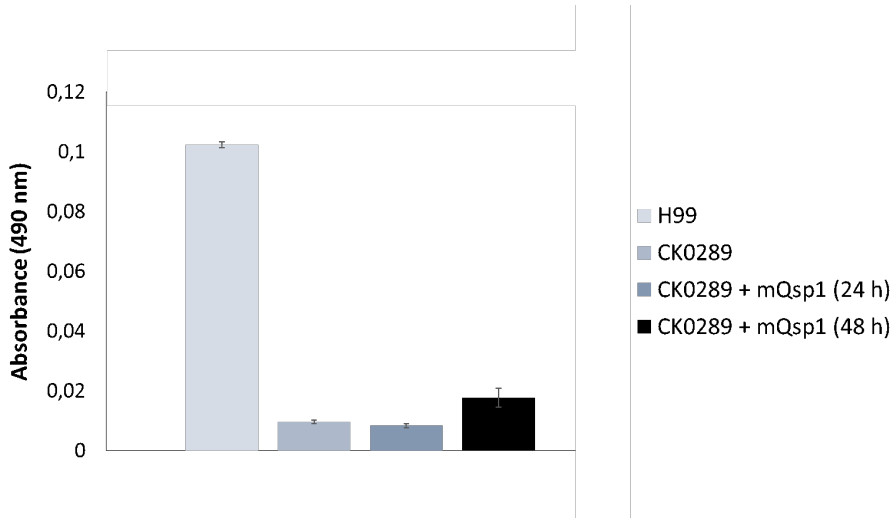

Figure 3.5: Melanisation of *Cryptococcus neoformans* strains grown in nitrogen-limiting medium with 0.2 g/L NH_4Cl (NLM-0.2) at 37°C with or without exposure to 80 µM synthetic mature Qsp1 (mQsp1). The quorums sensing mutant strain, *C. neoformans* CK0289 (qsp1Δ), was exposed to mQsp1 for either 24 h or 48 h, as indicated. Melanisation was recorded as the absorbance of melanin intermediates produced via laccase activity at a wavelength of 490 nm. Error bars represent standard error of the mean (n = 3).

Previous research has shown that the *C. neoformans* CK0289 QS mutant displayed a variable melanin phenotype depending on growth conditions (Homer et al., 2016). It is, therefore, likely that the observed phenotypes under NL and temperature stress are as a consequence of this variability and not necessarily due to a QS-specific response. Homer et al. (2016) found that Qsp1 regulated genes involved in cell wall biogenesis and that there were significant differences in the architecture of the cell walls of *C. neoformans* H99 and the *C. neoformans* CK0289 QS mutant. Because laccase

enzymes are mostly localised in the cell membrane of this yeast and melanin particles are deposited in the cell wall (Camacho et al., 2019), changes in the organisation of cell wall components of the QS mutant may have affected the distribution and activity of laccase enzymes.

In contrast to laccase activity, no urease activity was observed for each strain at 37°C when grown in both NLM (Fig. 3.6). This was unexpected given that the urease enzyme is required for *C. neoformans* dissemination and virulence in the human body (Cox et al., 2000; Olszewski et al., 2004; Singh et al., 2013), where the cells experience physiological temperature (37°C) and NL. Drastic pH changes in a cell's local environment can negatively affect its growth and survival. To cope with pH fluctuations, cells can release various metabolites that modify the pH of their surroundings (Vylkova, 2017). The assay used to quantify urease activity relies on measuring a yellow-to-pink colour change in media owing to a basic pH shift that would result when ammonia is released from urea degradation (Cox et al., 2000; Olszewski et al., 2004). Therefore, because the test relies on changes in pH, the assay is not specific to urea degradation and can thus be influenced by other factors that would affect the pH of the test media. During the combined stress of elevated temperature and NL, it is likely that *C. neoformans* may have released metabolites to neutralise the pH change caused by urea degradation, thus accounting for the lack of urease activity observed.

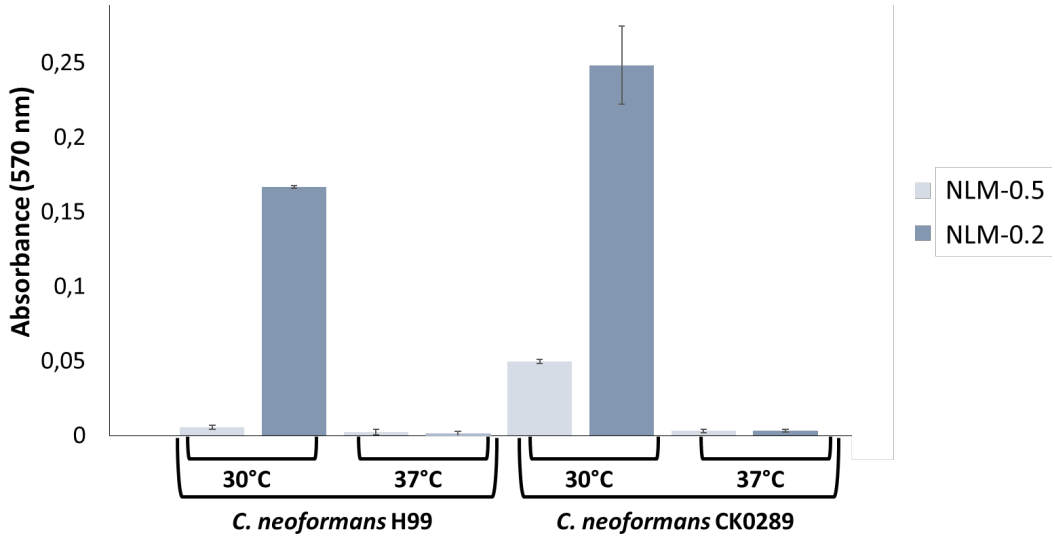

Figure 3.6: Urease activity of *Cryptococcus neoformans* strains grown under different nutrient-limiting and temperature conditions. Error bars represent standard error of the mean (n = 3).

Conversely, urease activity was detected for both strains at 30°C in NLM, where a decrease in nitrogen concentration induced a stronger response. In *C. neoformans*, nitrogen metabolism is tightly regulated by NCR (Kmetzsch et al., 2011; Lee et al., 2011). In the presence of limiting and non-preferred nitrogen sources, genes previously inhibited by NCR due to the availability of primary nitrogen sources are activated and promote the scavenging of alternative sources (Marzluf, 1997; Wong et al., 2008). This may explain why the activity for urease, a nitrogen scavenging enzyme that converts non-preferred urea to ammonia (Cox et al., 2000; Olszewski et al., 2004), showed a 31X

increase in NLM-0.2 (0.167±0.001) compared to NLM-0.5 (0.005±0.001) at 30°C for *C. neoformans* H99 ($P < 0.001$). Interestingly, *C. neoformans* CK0289 displayed a 10X and 1.5X higher absorbance value in NLM-0.5 (0.050±0.001) and NLM-0.2 (0.248±0.026) compared to the H99 strain ($P = 0.01$). The latter suggests that Qsp1 may be a negative regulator of urease activity. However, comparing the urease activity ratios for the individual strains between NLM-0.5 and NLM-0.2, *C. neoformans* H99 showed a 31X increase in urease activity compared to the 5X increase exhibited by *C. neoformans* CK0289. These differences between the strains imply that Qsp1 is possibly required for a heightened induction of urease activity during low levels of available nitrogen.

Exposure of *C. neoformans* CK0289 to the QS peptide did not recover the urease phenotype (Fig. 3.7). Instead, conflicting responses were observed. In NLM-0.5, 24 h and 48 h of exposure to 80 µM mQsp1 resulted in a significant increase in absorbance for the QS mutant (0.069±0.001 and 0.380±0.002, respectively) relative to the untreated control (0.050±0.001) ($P < 0.001$). Theoretically, it was expected that if urease activity were to be recovered after exposure to mQsp1, it would decrease to match that of the wild type H99 strain (0.005±0.001). Perhaps *C. neoformans* was degrading mQsp1 as well as possibly recycling its own proteins (autophagy) as a nitrogen source in response to NL (Ding et al., 2018; Soria and Brunetti-Pierri, 2019). This would result in more ammonia being released into the culture supernatant (Wolff et al., 1986), causing the test medium to exhibit a stronger colour change owing to an increase in pH. Consequently, higher absorbance would be measured. However, no marked difference in urease activity was observed between the *C. neoformans* CK0289 QS mutant exposed and unexposed to mQsp1 in the even more nitrogen-deficient medium, NLM-0.2 (Fig. 3.7). Here, urease activity was again expected to decrease to match that of the H99 strain (0.050±0.001). After 48 h of exposure to mQsp1, urease activity of the mutant strain does appear to decrease (0.225±0.002) relative to the untreated control (0.248±0.026), however, this was not significant ($P = 0.65$). Altogether, these results suggest that Qsp1 does not regulate the expression of urease enzymes and that other non-QS mechanisms induced in response to NL are in play. However, a more urease-specific assay should be done to eliminate the potential variable of other factors influencing these results. Moreover, the test medium could be optimised to include a buffer in order to mitigate the possible interference with assay measurements due to released organic acids by the cells.

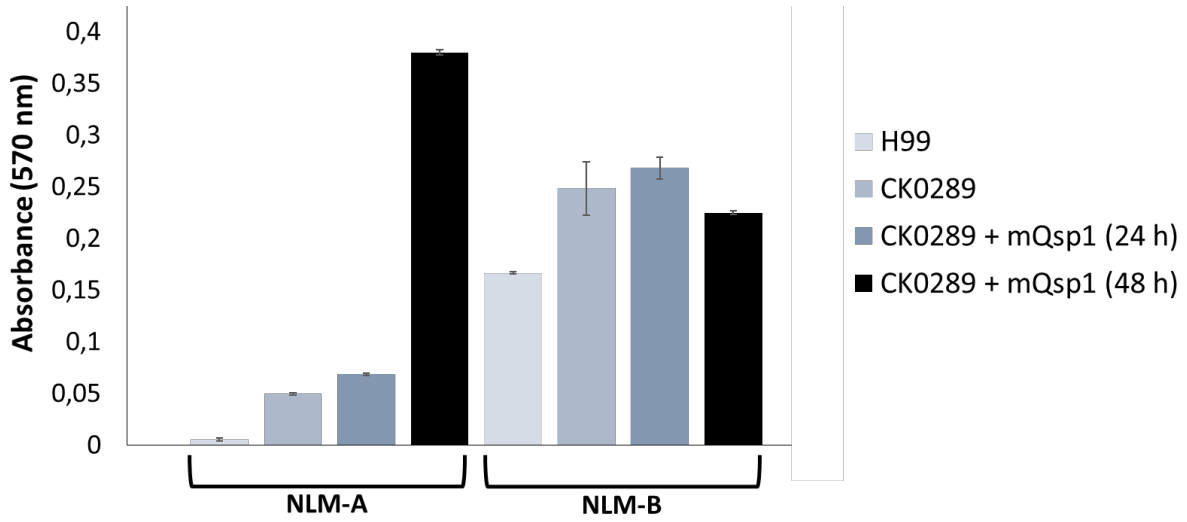

Figure 3.7: Urease activity of *Cryptococcus neoformans* strains grown in different nutrient-limiting conditions at 30°C with or without exposure to 80 µM synthetic mature Qsp1 (mQsp1). The quorum sensing mutant strain, *C. neoformans* CK0289 (qsp1Δ), was exposed to mQsp1 for either 24 h or 48 h, as indicated. Error bars represent standard error of the mean (n = 3).

3.4.3 In addition to nitrogen and temperature, Qsp1 may also regulate ergosterol biosynthesis

The combined factors of NL and the elevated temperature was found to play a strong role in regulating the ergosterol content in the cell membrane of *C. neoformans*. It was found that *C. neoformans* H99 produced a 2X higher percentage of ergosterol in its total cell content when grown in NLM-0.2 with high-temperature stress (0.0052±0.0003%) compared to growth in NLM-0.5 (0.0026±0.0002%) ($P < 0.001$) (Fig. 3.8). This confirms previous observations reported by Bosch et al. (unpublished) for various *C. neoformans* and *C. gattii* strains tested under NL. A similar trend under these specific conditions was presented when testing melanisation (Fig. 2.4). Moreover, our data also confirms previous findings that suggested that NL affects ergosterol biosynthesis via regulation by the nitrogen-sensing Gat1 transcription factor (Kmetzsch et al., 2011).

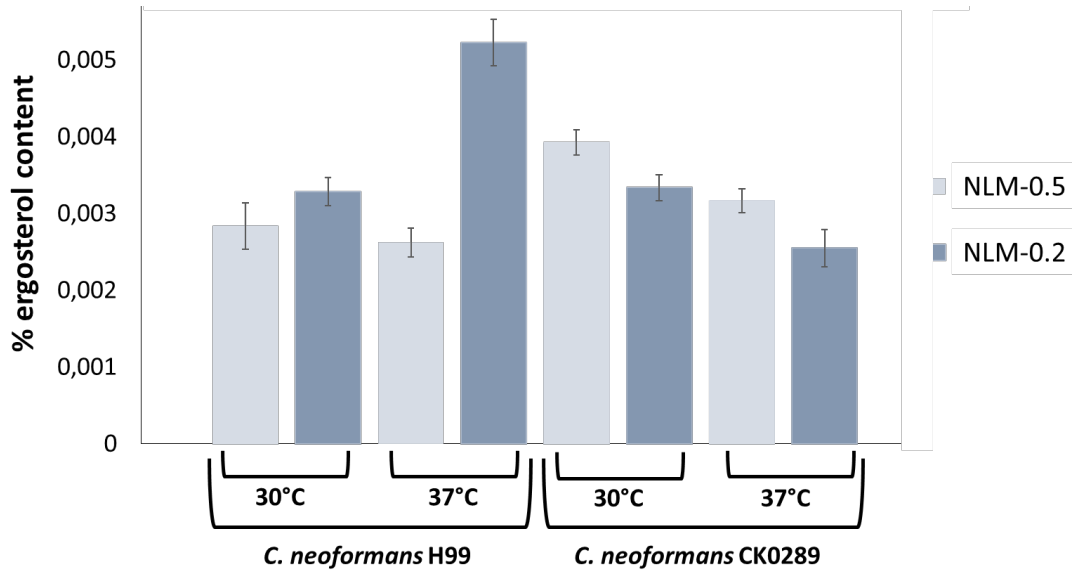

Figure 3.8: Ergosterol content as mean percentage wet pellet weight determined for *Cryptococcus neoformans* strains grown under different nitrogen-limiting and temperature conditions. Error bars represent standard error of the mean (n = 3).

Under the same conditions of low nitrogen and high-temperature stress, however, *C. neoformans* CK0289 did not show a significant increase in ergosterol content. This suggests that Qsp1 could be necessary for controlling rapid ergosterol biosynthesis when cellular stress is high. Qsp1 may be a regulator of stress-activated HOG signalling and could play an important role in how the cell copes in response to nutrient and temperature stress when cell densities are high. Although ergosterol is not a cryptococcal virulence factor, it is an essential part of the yeast cell membrane, being responsible for membrane integrity and fluidity (Parks and Casey, 1995). Temperature stress has previously been implicated in the regulation of ergosterol biosynthesis in yeast (Shang et al., 2006; Bahn et al., 2007). However, NL and QS have not been implicitly studied in regulating ergosterol biosynthesis in *C. neoformans*. Due to the nature of the ergosterol assay, a direct link between the observed phenotype and QS via Qsp1 could not be made because high volumes of mQsp1 would be required to perform a phenotype recovery assay similar to that performed for the cryptococcal virulence factors.

3.5 Conclusion

Changes in QS responses in conditions of NL have not been explicitly investigated for *C. neoformans*. This study highlights the role NL plays in regulating the virulence phenotype of *C. neoformans* and that this may also be controlled by mechanisms involving QS during specific temperature conditions. However, this still needs further investigation as this study primarily compared phenotypic changes observed between a wild type and a QS mutant strain. Direct correlations to the pathogenesis of *C. neoformans* in relation to NL in mammalian hosts cannot be made as ammonia concentrations in human blood plasma and cerebrospinal fluid, as examples, is usually maintained at concentrations around 40-50 µM (Walker, 2014) and 12 µM (Lee et al., 2013; The Human Metabolome Database 4.0, http://www.hmdb.ca/), respectively. These concentrations are much lower than that tested in this study – 9.3 mM in NLM-0.5 and 3.7 mM in NLM-0.2. However, one can still infer that limiting nitrogen could play a strong role in affecting the virulence of *C. neoformans* during infection. Ecologically relevant concentrations of nitrogen were used in this study, which resulted in the pronounced expression of cryptococcal virulence factors that also promote survival. This suggests that environmental stressors may have primed *C. neoformans* for pathogenesis as a consequence of adaptations developed to survive the harsh conditions of its natural environment. All tested cryptococcal virulence factors are known to be regulated by stress-activated signalling pathways, such as the target of rapamycin (TOR) and cyclic adenosine monophosphate-protein kinase A (cAMP-PKA) pathways, which respond to NL (Cramer et al., 2006; Lee et al., 2011; Ries et al., 2018). Once favourable nitrogen sources are depleted these nitrogen sensing pathways, together with the GATA-type transcription factor, Gat1 (Kmetzsch et al., 2011;

Lee et al., 2011), induces the expression of important genes involved in alternative nitrogen source assimilation, virulence and ergosterol biosynthesis. Since the virulence phenotype of *C. neoformans* and ergosterol biosynthesis was affected by NL as well as Qsp1, this suggests that QS may potentially interact with Gat1 and help regulate these stress-activated signalling cascades. Characterisation of this potential interaction could help further improve the understanding of the mechanisms involved in regulating the pathogenicity of *C. neoformans*.

Because Qsp1 potentially influenced the cryptococcal capsule, it could serve as a promising antipathogenic target. Using an antivirulence approach in drug design and in novel treatment strategies, which targets microbial components not essential for cell survival (Fong et al., 2017), could help overcome the crisis of antifungal drug resistance because the probability of pathogens rapidly acquiring resistance is reduced (Cegelski et al., 2008; Jakobsen et al., 2012; LaSarre and Federle, 2013). Current limitations to using this strategy against *C. neoformans* are the lack of understanding of the genetic and physiological factors involved in cryptococcal QS as well as the fact that no literature has reported on potential compounds that directly interfere with cryptococcal QS. This makes drug target design challenging and, although quorum quenching drugs against other pathogens can be prioritised for screening, most of these tests would be performed blind. Regardless, concerted effort in unravelling the cryptococcal QS response will prove valuable and may aid in various fields of *Cryptococcus* research.

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CHAPTER 4

RESEARCH RESULTS II

Antivirulent activity of garlic-derived organosulfur compounds against *Cryptococcus neoformans*

Chapter 4: Antivirulent activity of garlic-derived organosulfur compounds against *Cryptococcus neoformans*

4.1 Abstract

Cryptococcus neoformans infections in immune-compromised mammalian hosts eventually lead to the development of life-threatening fungal meningitis. The yeast produces a range of virulence factors that enable it to cause disease during host infection. By inhibiting the expression of these virulence factors, and quorum sensing (QS) responses involved in their regulation, may check disease progression. Because virulence factors and QS are not essential for yeast survival, it is thought that drugs that target these components may have prolonged effective life spans as acquired drug resistance may be limited. Organosulfur compounds derived from garlic (*Allium sativum* L.) have previously shown potent antivirulent activity against *Pseudomonas aeruginosa*, inhibiting both virulence factor production and QS mechanisms. To date, no drugs have been reported to inhibit cryptococcal QS and consequently affect virulence factor expression. Here we provide the first report of the anticryptococcal activity of ajoene, as well as the effect of ajoene and allicin on selected *C. neoformans* virulence factors. Natural garlic disulfides showed more potent minimum inhibitory concentrations than synthetic analogues. Regarding antipathogenic activity, ajoene and allicin significantly affected capsule enlargement and completely inhibited melanisation. The natural organosulfides did not inhibit urease activity and ergosterol biosynthesis. Compared to *C. neoformans* H99, a *C. neoformans* CK0289 (qsp1Δ) QS mutant showed higher resistance to most organosulfides tested. Preliminary data suggest that the effect these compounds have on cryptococcal virulence factors are likely not due to interference with Qsp1-regulated QS responses. Overall, garlic-derived organosulfides are promising antipathogenic drugs which may provide alternative solutions to treating *C. neoformans* infections.

4.2 Introduction

Cryptococcus neoformans infections in immune-compromised individuals result in cryptococcosis, a detrimental disease that leads to the development of fungal meningitis (Saag et al., 2000; Perfect and Bicanic, 2015). Infections by this dimorphic fungus occur once airborne spores are inhaled and deposited in the lungs (Buchanan and Murphy, 1998). After the infection is established, cryptococcal cells can disseminate from the lungs into the bloodstream, whereafter they are able to cross the blood-brain barrier and aggregate in the brain (Santiago-Tirado et al., 2017). The accumulation of *C. neoformans* in the brain, followed by the swelling of brain tissue, is responsible for the onset of meningitis. Despite the development of effective treatment solutions, cryptococcal meningitis kills 180000 people annually, where 75% of these deaths occur in sub-Saharan Africa (Brodt et al., 1997; Offiah and Naseer, 2016; Rajasingham et al., 2017).

Currently, the most common drugs used for anticryptococcal treatment include fluconazole (FLC), amphotericin B (AmB) and flucytosine. The drugs, FLC and AmB, disrupt fungal cell membrane integrity by targeting ergosterol, an important fungal sterol (Holz, 1974; Bossche et al., 2009), and the antimetabolite drug, flucytosine, inhibits growth by interfering with fungal DNA and RNA synthesis (Vermes et al., 2000). The World Health Organisation recommends a two-week-long AmB and flucytosine combination therapy, followed by treatment with FLC to clear the infection (Smith et al., 2015). Because FLC is fungistatic, it is not recommended for use in monotherapy (Larsen et al., 1990; Schaars et al., 2006). Although AmB and flucytosine have shown effective clearance of *C. neoformans* infections (Molloy et al., 2018), they have significant drawbacks. Most AmB formulations can cause renal toxicity and are expensive because it requires intravenous administration (Perfect and Bicanic, 2015). In regions with a high prevalence of individuals susceptible to cryptococcosis, such as sub-Saharan Africa, flucytosine remains largely unavailable (Perfect and Bicanic, 2015; Azevedo et al., 2016). Moreover, *C. neoformans* has been reported to acquire resistance to these antifungal drugs if they are used for a prolonged period of time (Gago et al., 2016). This, therefore, highlights the need for alternative treatment solutions where novel anticryptococcal strategies are employed or new, effective and easily accessible antifungal drugs are developed.

The ability of *C. neoformans* to successfully infect immunocompromised mammalian hosts and cause cryptococcal meningitis is attributed to its production of surfeit virulence-associated factors (Malachowski et al., 2016). Some of the most well-studied cryptococcal virulence factors include the polysaccharide capsule (Bulmer et al., 1967; Zaragoza et al., 2009), melanin deposits in the cell wall (Wang et al., 1996; Rosas et al., 2000), and urease activity (Cox et al., 2000; Olszweski et al., 2004). *Cryptococcus* strains with defects in these virulence factors have presented reduced virulence in mouse infection models (Kwon-Chung et al., 1982; Chang et al., 1996; Fu et al., 2018). Inhibition of these virulence factors could thus serve as a feasible strategy to treat cryptococcal infections. Moreover, they are promising drug targets because laccases, ureases and polysaccharide components of the cryptococcal capsule are absent in mammalian hosts (Rutherford, 2014; Azevedo et al., 2016). Microbial virulence factors are generally known to be regulated by small, secreted signalling molecules (autoinducers) in a process called quorum sensing (QS) (Bandara et al., 2012). Although not extensively studied, QS via a small peptide (Qsp1) (Lee et al., 2007) was found to regulate virulence in *C. neoformans* (Homer et al., 2016). Hence, interfering with QS presents another potential antivirulence strategy against *C. neoformans*. In theory, using an antivirulence approach would reduce the likelihood of a pathogen acquiring drug resistance because non-essential components are targeted (Cegelski et al., 2008; Jakobsen et al., 2012; LaSarre and Federle, 2013; Kirienko et al., 2019). Consequently, weaker selection pressure would be imposed compared to drugs that inhibit the growth of or kill a microorganism. This is especially critical since fungal pathogens have acquired resistance to most antifungal drugs available and no new antifungal drug

classes cogent against *C. neoformans* have been developed since the introduction of fluconazole (FLC) in the 1990s (Perfect, 2017).

Tuber and aerial tissue extracts of *Pelargonium sidoides*, a South African medicinal herb, were found not only to constrain the growth of *C. neoformans* but also to reduce capsule thickness and inhibit laccase and urease activity (Samie et al., 2019). This study demonstrated that medicinal herbs might provide a new source of drugs with antivirulent potential against *C. neoformans*. The garlic bulb (*Allium sativum* L.) is well known for its many health benefits and has been used as a medicine since 1550 B.C. in ancient Egypt (Garty, 1993). Garlic extracts and major garlic compounds, such as ajoene, have shown antimicrobial, virulence attenuating and quorum quenching (QQ) activity against *Pseudomonas aeruginosa* (Bjarnsholt et al., 2005; Jakobsen et al., 2012). The bioactivity of ajoene is largely attributed to its terminal allyl and central vinyl disulfide functional groups (Gallwitz et al., 1999). In accordance, an array of disulfide-containing analogues also exhibited antipathogenic properties (Fong et al., 2017). Modifying the terminal allyl ends of ajoene with other functional groups appeared to improve the stability and bioactivity of the compound (Hunter et al., 2008). In light of this, designing drug analogues that contain disulfide functional groups with various structural organisations could be important in antipathogenic drug discovery research. Garlic extracts and a natural garlic thiosulfinate, allicin, show antifungal activity against *C. neoformans* (Yamada and Azuma, 1977; Fromtling and Bulmer, 1978). Currently, no drugs have been reported to inhibit QS for this pathogen. Since disulfide-containing compounds have antipathogenic activity against bacteria, and these compounds have shown inhibitory activity against *C. neoformans*, it seemed plausible to screen these compounds for potential cryptococcal virulence attenuation and QQ activity.

In this study, we aimed to investigate whether garlic-derived organosulfur compounds could affect the expression of QS-regulated virulence factors. These virulence factors included the polysaccharide capsule, melanisation (laccase activity) and urease activity. Due to previous findings suggesting that QS may influence ergosterol content in the cell membrane of *C. neoformans* (Chapter 3, section 3.4.3), we also tested whether these compounds could affect ergosterol biosynthesis.

4.3 Materials and methods

All reagents were supplied by Merck (Darmstadt, Germany) unless otherwise indicated.

4.3.1 Yeast strains

A *C. neoformans var. grubii* H99 (serotype A) strain was kindly provided by Dr. Joseph Heitman (Duke University, Durham, NC), and a *C. neoformans* CK0289 QS mutant (qsp1Δ; H99 background) strain was a kind gift from Prof. Hiten Madhani (University of California, San Francisco, CA) (Homer et al., 2016).

4.3.2 Media

Yeast extract-peptone-dextrose (YPD) medium was prepared with 20 g/L peptone, 10 g/L yeast extract and 20 g/L glucose. Solid YPD medium contained 15 g/L agar. Nitrogen-limiting media (NLM) was prepared using 11.7 g/L Yeast Carbon Base (BD Life Sciences, New Jersey, USA) with 0.2 g/L NH_4Cl (NLM-0.2). The pH was adjusted to 5.5 with NaOH.

4.3.3 Antifungal activity of garlic-derived organosulfur compounds

The minimum inhibitory concentration (MIC) of a compound was determined using a colourimetric assay (Samie et al. 2019) with slight modifications – NLM-0.2 was used during all steps following the preparation of overnight yeast cultures. Inhibitors included organic disulfide and trisulfide compounds (Table 4.1), as well as 0.02 mg/mL cycloheximide as a positive control. The organosulfides were kindly provided by Dr. Catherine Kaschula (Department of Chemistry, Stellenbosch University, South Africa). Compounds D1-D6 and D9 are synthetic analogues of the natural, garlic-derived compound, D7 (*E*/*Z*-ajoene). D8 (allicin) is a natural garlic compound, while T1 and T2 are analogues of diallyl trisulfide (DATS). Note that DATS was not included in this study. Organosulfur compounds were dissolved in 100% (v/v) dimethylsulfoxide (DMSO) and serially diluted, with concentrations ranging from 1.28 mM to 5 µM. Data were reported in µg/mL as is convention for these assays. All treatments were performed in triplicate. Untreated, as well as media sterility and heat-killed cells (95°C, 1 h) were included as controls. Additionally, DMSO was added as a vehicle control at concentrations ranging from 2.56-0.01% (v/v). The lipophilicity index (LogP) of each compound in Table 4.1 was calculated using ChemDraw software (version 19.0, PerkinElmer, Massachusetts, USA). The higher the LogP value, the more lipophilic and thus hydrophobic (Arnott et al., 2013).

4.3.4 Antivirulence assays using natural organosulfides

4.3.4.1 Preparation of yeast inocula

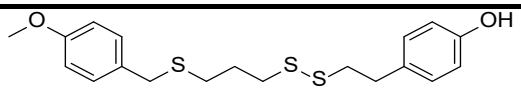
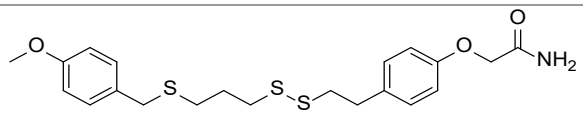
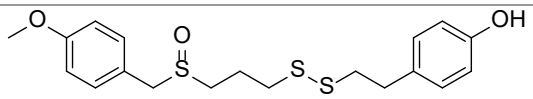
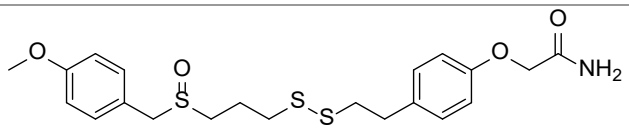
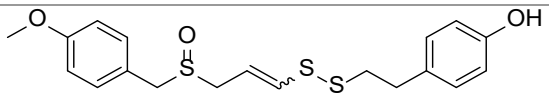
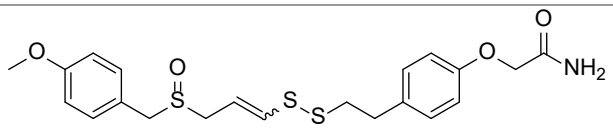
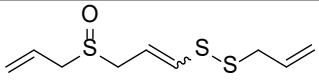
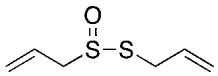
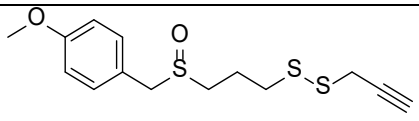
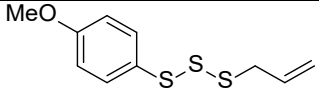
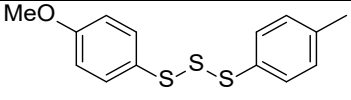
Yeast cultures were prepared in test tubes by inoculating a single yeast colony into 5 mL YPD broth and incubated for 24 h at 30°C on a rotating wheel. Cells were harvested, washed twice and resuspended in sterile phosphate-buffered saline (PBS) solution before transfer to test medium at specified cell densities. All wash steps were performed with PBS, and harvesting was done using centrifugation (5000 x *g*, 1 min) unless stated otherwise.

4.3.4.2 Polysaccharide capsule thickness measurements

Washed yeast cells were inoculated to a cell density of 1 x 10^6 cells/mL in 5 mL NLM-0.2 dosed with a sub-inhibitory concentration of ajoene or allicin. Untreated samples contained 0.16 % (v/v) DMSO in media. Cells were grown to saturation for 48 h at 37°C on a rotating wheel. Following incubation, the cells were harvested, washed twice and stained with 10% (w/v) nigrosin before capturing images of the encapsulated cells with a Nikon Eclipse E400 fluorescence microscope (Laboratory Optical

Service, Inc., Virginia, USA). Yeast cell radius and total radius including the capsules were measured for 80-100 cells per treatment using ImageJ 1.51 j8 software (National Institutes of Health, Bethesda, USA) (Frases et al., 2009). Capsule thickness was determined by subtracting the yeast cell radius from the total radius. All treatments were performed in triplicate and data normalised against mean cell diameter.

Table 4.1: Organosulfur compounds used in the *Cryptococcus neoformans* antifungal and virulence attenuating assays

Compound	Chemical structure	Molecular weight (g/mol)	LogP
Disulfides			
D1		380.58	5.47
D2		437.63	4.35
D3		396.58	3.52
D4		453.63	2.39
D5		394.56	3.82
D6		451.61	2.70
D7 (*E*/*Z*-ajoene)		234.39	1.97
D8 (Allicin)		162.27	1.35
D9		314.49	2.11
Trisulfides			
T1		244.38	4.56
T2		294.44	6.02

4.3.4.3 Laccase activity

Laccase activity was tested using an approach described by Eisenman et al. (2007) with modifications. Washed yeast cells were inoculated to a cell density of 1 x 10^6 cells/mL into test tubes containing 5 mL NLM-0.2 containing a sub-inhibitory dose of ajoene or allicin. Untreated samples contained 0.16 % (v/v) DMSO in media. Cells were incubated to saturation for 48 h at 37°C on a rotating wheel. After that, cultures were harvested, washed twice and inoculated to a cell concentration of 5 x 10^6 cells/mL in test tubes containing 5 mL reverse osmosis-purified (RO) water with 5 mM 3,4-dihydroxy-L-phenylalanine (L-DOPA, TLC grade). Samples were incubated at 37°C in the dark on a rotating wheel. Absorbance of each sample was measured after 24 h at a wavelength of 490 nm. All treatments were performed in triplicate and a control included cells without L-DOPA.

4.3.4.4 Urease activity

Assays were performed in the same manner as described for laccase activity. After saturated growth in NLM-0.2 containing sub-lethal concentrations of ajoene or allicin, cells were harvested, washed twice with RO water and resuspended to a cell concentration of 5 x 10^6 cells/mL in test tubes containing 5 mL assay medium. The assay medium contained 0.66 mM urea as a substrate and 0.012 g/L phenol red pH indicator. Samples were incubated at 30°C on a rotating wheel. An absorbance reading of each sample was taken after 24 h at a wavelength of 570 nm. Untreated samples contained 0.16 % (v/v) DMSO in media. The experiment was performed in triplicate, and a control containing only cells and indicator (no substrate) was included.

4.3.4 Inhibition of ergosterol biosynthesis

The method of fungal ergosterol quantitation, as described by Samie et al. (2019) was performed with modifications. These included inoculating cells into 250 mL conical flasks containing 25 mL NLM-0.2 and a sub-lethal dose of allicin. For each treatment, two separate flasks with 25 mL media were inoculated using the same colony suspension (after harvesting, these were combined to obtain a final culture volume of 50 mL). Flasks were incubated at 37°C for 48 h on an orbital shaker (120 rpm) followed by harvesting (centrifugation at 2800 x *g* for 6 min) and washing with sterile RO water. Wet pellet weight measurements were taken as described previously. Untreated samples contained 0.16 % (v/v) DMSO in media and a positive control included treatment with 8 µg/mL FLC. Each treatment was performed in triplicate.

4.3.5 Data visualisation and statistical analyses

All box and scatter plots were generated using an online software tool available at https://gabrifc.shinyapps.io/raincloudplots/ (Allen et al., 2019). Mean values were plotted with error bars denoting the standard error of the mean. For all datasets, the analysis of variance (ANOVA) was measured using one-way ANOVA, where the mean values were compared using Tukey's

honestly significant difference post hoc test for multiple comparisons with a significance level of 0.05. Statistical analyses were performed using Statistica (version 13, TIBCO Software Inc., USA).

4.4 Results and discussion

The nitrogen-limiting minimal medium, NLM-0.2, was used for most experiments in order to induce virulence factor expression and the stress of NL. Moreover, it is recommended that antifungal assays be performed using minimal media as opposed to rich media because they simulate stressful conditions likely experienced by a microorganism in its environment or during host infection (Rabjohns et al., 2013). The NLM-0.2 medium contains a nitrogen concentration of 0.2 g/L, less than half of the nitrogen concentration previously quantified from the woody substrates of oak trees situated in Stellenbosch Central (South Africa) upon which *C. neoformans* was isolated (Vreulink et al., unpublished). This medium was used to represent a fluctuation in nitrogen concentration likely experienced by *C. neoformans* in its environment.

4.4.1 Natural garlic organosulfides show strong antifungal activity against *C. neoformans*

Various organosulfur compounds derived from garlic were tested for antifungal activity against *C. neoformans*. The natural garlic compounds, ajoene and allicin, displayed potent anticryptococcal activity compared to all tested compounds (Table 4.2). The MIC of allicin against *C. neoformans* H99 was 2-17X higher than what has previously been reported in the literature. Allicin was previously found to inhibit several *C. neoformans* strains at concentrations between 1.57-12.5 µg/mL (Yamada and Azuma, 1977), which varied depending on the alkalinity of the test medium and inoculum size. However, the authors did use a rich growth medium as opposed to a minimal medium used in this study. This emphasises the effect growth conditions have on the bioactivity of drugs. Growth conditions affect the physiology of the organism being studied (Haukeli and Lie, 1971), which will influence the way it responds to inhibitory drugs. *Cryptococcus* generally increases the expression of virulence factors that protect against antimicrobial drugs during nutrient starvation, such as the polysaccharide capsule and melanin (Liu et al., 1999; Zaragoza et al., 2008; Cordero and Casadevall, 2017). This may explain the higher MIC obtained in the current work. Therefore, understanding how *C. neoformans* behaves under various nutrient conditions, especially the way it modulates its virulence phenotype, will greatly improve strategies employed to treat infections.

Despite allicin and garlic extracts having been tested against *C. neoformans* previously (Yamada and Azuma, 1977; Davis et al., 1990; Khan and Katiyar, 2000; Rehman and Mairaj, 2013), the inhibitory activity of ajoene has not been characterised. Therefore, this study provides the first report of the antifungal activity of purified ajoene against *C. neoformans*. Ajoene has, however, been tested against other fungi and showed more potent inhibitory activity, with MIC values 2-5X lower than that obtained for *C. neoformans* H99 in this study (Table 4.2). Yoshida and co-workers (1987)

demonstrated that ajoene inhibited the growth of *Candida albicans* and *Aspergillus niger* at concentrations of 7.6 µg/mL and 16.6 µg/mL, respectively. Also, a concentration of 11.7 µg/mL ajoene was found to inhibit the growth of *Paracoccidioides brasiliensis* (San-Blas et al., 1989). The higher MIC obtained for *C. neoformans* in this study compared to other fungi may be attributed to its ability to produce various protective layers, such as the polysaccharide capsule and melanin, which are known to reduce the efficacy of antimicrobial drugs (Liu et al., 1999; Zaragoza et al., 2008; Cordero and Casadevall, 2017).

Table 4.2: Minimum inhibitory concentrations (MICs) of selected organosulfur compounds against *Cryptococcus neoformans* strains

	D4	D7	D8	D9
Strain	**Concentration (µg/mL)**			
C. neoformans H99	72.6	37.5	26	402.5
C. neoformans CK0289	/	75	104	201

/ = no antifungal activity

Most synthetic organosulfur analogues (D1-D3, D5-D6 and T1-T2) exhibited no antifungal activity against both *C. neoformans* strains tested (data not shown). Only two synthetic disulfide derivatives of ajoene (D4 and D9) showed antifungal activity, but at higher concentrations than the natural compounds (Table 4.2). Ajoene is known to lose stability and activity after incubation for 24 h at 37°C (Jakobsen et al., 2012), while allicin rapidly degrades at temperatures of 40°C and above (Wang et al., 2015). Because compounds D1-D6 and D9 are synthetic analogues of ajoene, and similar in base structure to allicin, they may also be temperature sensitive. The antifungal susceptibility assays were performed at 37°C for 24 h, which might have resulted in a short half-life for the analogues and thus no bioactivity was detectable. This could also explain why higher MICs were reported for ajoene (37.5 µg/mL) and allicin (26 µg/mL) compared to those reported in the literature. Furthermore, the synthetic analogues contained bulky side chains, which increases their hydrophobicity and reduces their solubility. This was observed for all synthetic analogues, even D4 and D9, which precipitated when diluted in test media.

The limited solubility of most of the compounds was confirmed by their estimated LogP values (Table 4.1). Ajoene and allicin, which did not precipitate in solution, had the lowest LogP values, confirming their water solubility. Despite compounds D4 and D9 precipitating in solution, they also displayed

low LogP values, suggesting that they likely did not precipitate to the same degree as the other synthetic derivatives. Altogether, the low LogP values calculated for D4, ajoene, allicin and D9 could explain why they were the only compounds displaying bioactivity during each treatment.

Interestingly, *C. neoformans* CK0289 survived higher doses of the test compounds (Table 4.2). It was expected that the QS mutant would be more susceptible to inhibitory drugs as its QS mechanism, which regulates protective virulence factor expression, was impaired. Kaschula et al. (2019) showed that ajoene localised in the endoplasmic reticulum of cancer cells and targeted exposed cysteine residues in newly synthesised proteins. Many of these newly formed targeted proteins misfolded, which activated the unfolded protein response and eventually caused apoptosis. Theoretically, more actively growing cancer cells would have higher levels of mRNA synthesis and protein production (García-Martínez et al., 2016). These cells would, therefore, be more susceptible to the inhibitory action of ajoene. In microbes, QS stimulates a cell density-dependent boost in transcription and the synthesis of various proteins (Taillefumier and Wingreen, 2015). Because *C. neoformans* CK0289 lacks this mechanism, it likely does not present an increase in protein production during saturated growth compared to *C. neoformans* H99. It is possible that the organosulfides would not be as toxic to the *C. neoformans* CK0289 QS mutant, which could explain its higher resistance to these drugs. However, growth studies are needed to be done to confirm this. Additionally, microscopic analyses coupled with fluorescent ajoene need to be performed to confirm whether ajoene and its derivatives have a similar mechanism of action by localising in the yeast endoplasmic reticulum.

Moreover, the *C. neoformans* CK0289 QS mutant's cell wall is structurally different to that of the wild type strain (Homer et al., 2016). This could potentially affect how the compounds interact with and penetrate the cell wall. The movement of fluorescent-labelled organosulfur compounds could be used to monitor whether there is a difference in the rate of uptake as well as intracellular accumulation of these compounds, which would likely explain the observed differences in MICs between the H99 wild type strain and CK0289 QS mutant. Although most of the synthetic compounds show no antifungal activity, they may still have antivirulence potential by interfering with virulence factor expression and QS.

4.4.2 Antivirulence activity of natural garlic-derived organosulfides

As the virulence attenuation assays served to indicate the *C. neoformans* antipathogenic potential of ajoene and allicin, and no comparisons between nutrient and temperature conditions were made, all assays were performed under conditions that previously showed the strongest virulence phenotype (Chapter 3). Moreover, a drug concentration of half the MIC was defined as being sub-inhibitory. Therefore, all treatments were performed using 19 µg/mL ajoene and 13 µg/mL allicin.

4.4.2.1 Ajoene and allicin affect the capsule phenotype and cellular morphology of *C. neoformans*

Treatment with 13 µg/mL allicin significantly reduced the capsule thickness of *C. neoformans* H99 (0.249±0.004 µm) relative to the untreated control (0.338±0.004 µm) ($P < 0.001$) (Fig. 4.1). Similarly, the treated *C. neoformans* CK0289 QS mutant also displayed significantly thinner capsules (0.221±0.004 µm) than the untreated control (0.278±0.004 µm) ($P < 0.001$). Because allicin reduced the capsule thickness of both strains, this suggests that inhibition of capsule enlargement was not due to QQ activity. If allicin inhibited capsule enlargement by interfering with QS specifically, the compound should theoretically not have any effect on the capsule phenotype of the *C. neoformans* CK0289 QS mutant and only reduce the capsule thickness of the H99 wild type cells. Therefore, allicin could be interfering with either capsule biosynthesis or directly damaging the structure of the capsule. The significant reduction in capsule thickness observed still motivates for allicin as a potential *Cryptococcus* antipathogenic agent.

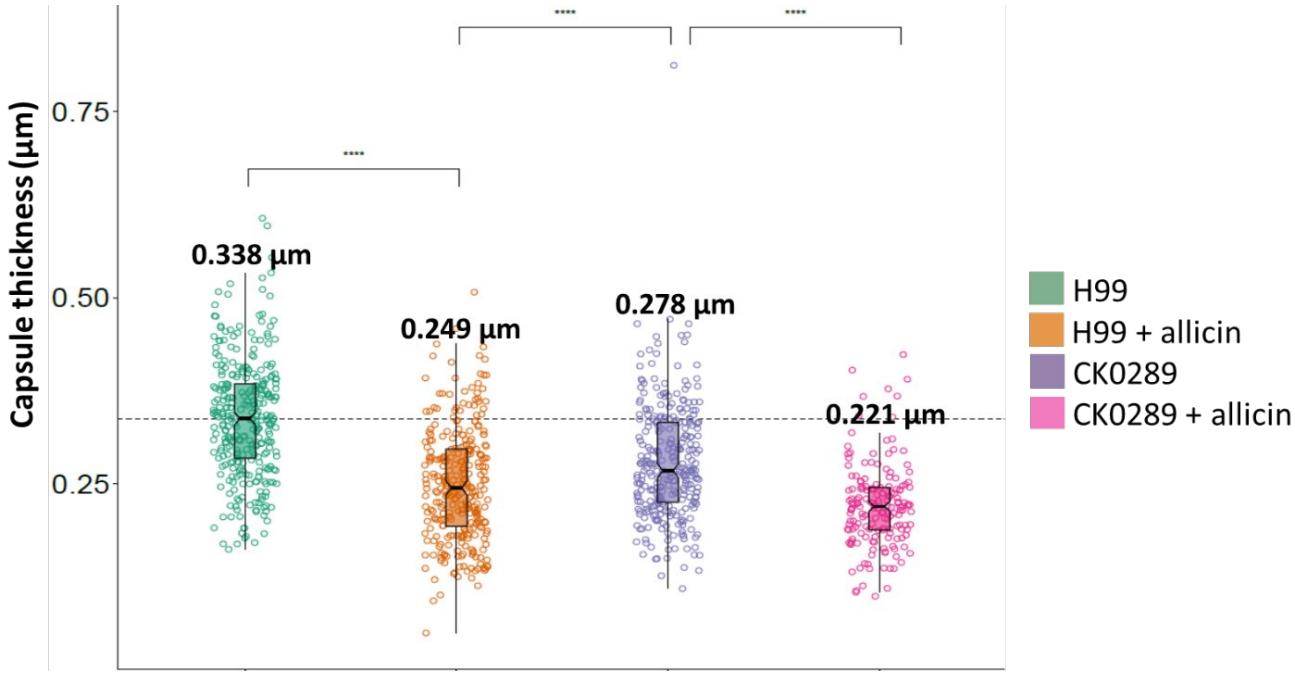


Figure 4.1: Capsule thickness of *Cryptococcus neoformans* strains grown in nitrogen-limiting media with 0.2 g/L NH_4Cl (NLM-0.2) at 37°C with or without exposure to 13 µg/mL allicin. The average capsule thickness for each condition is indicated above each boxplot. The dashed line indicates the average capsule thickness for the untreated *C. neoformans* H99 wild type strain (0.338±0.004 µm).

Strikingly, ajoene exposure (19 µg/mL) appeared to induce both cryptococcal strains to transition from yeast to filamentous growth morphologies (Fig. 4.2). Most cells (~80%) observed were in their pseudohyphal state with only a few yeast cells visible; hence, capsule thickness could not be measured. This was an interesting observation because ajoene was previously found to severely damage hyphal structures of *A. niger* (Yoshida et al., 1987). Moreover, ajoene caused a delay in the transition of *P. brasiliensis* yeast cells to hyphal form (San-Blas et al., 1993), although all cells were able to switch by 48 h of growth. Many fungi display increased pathogenesis after the yeast-to-hyphal

transition as filamentous growth facilitates tissue invasion and dissemination (Trevijano-Contador et al., 2016). Although *C. neoformans* does have the capacity to produce hyphal and pseudohyphal structures (Lin et al., 2016), it is predominantly found in its yeast form during infection of mammalian hosts. The hyphal and pseudohyphal forms of *C. neoformans* are also less virulent than its yeast form in mammalian hosts but confer resistance against amoeboid phagocytosis (Neilson et al., 1978) in its natural environment. Because pseudohyphae were previously found during some cases of human host infection (Gazzoni et al., 2009; Gazzoni et al., 2010) and predation by amoebae (Neilson et al., 1978), this suggests that *C. neoformans* may undergo morphological transitioning to protect itself from specific stressors. It could thus be likely that during growth in NLM with elevated temperature (37°C), the added stress of ajoene exposure may have caused the cells to produce pseudohyphae for protection. However, this response needs further investigation.

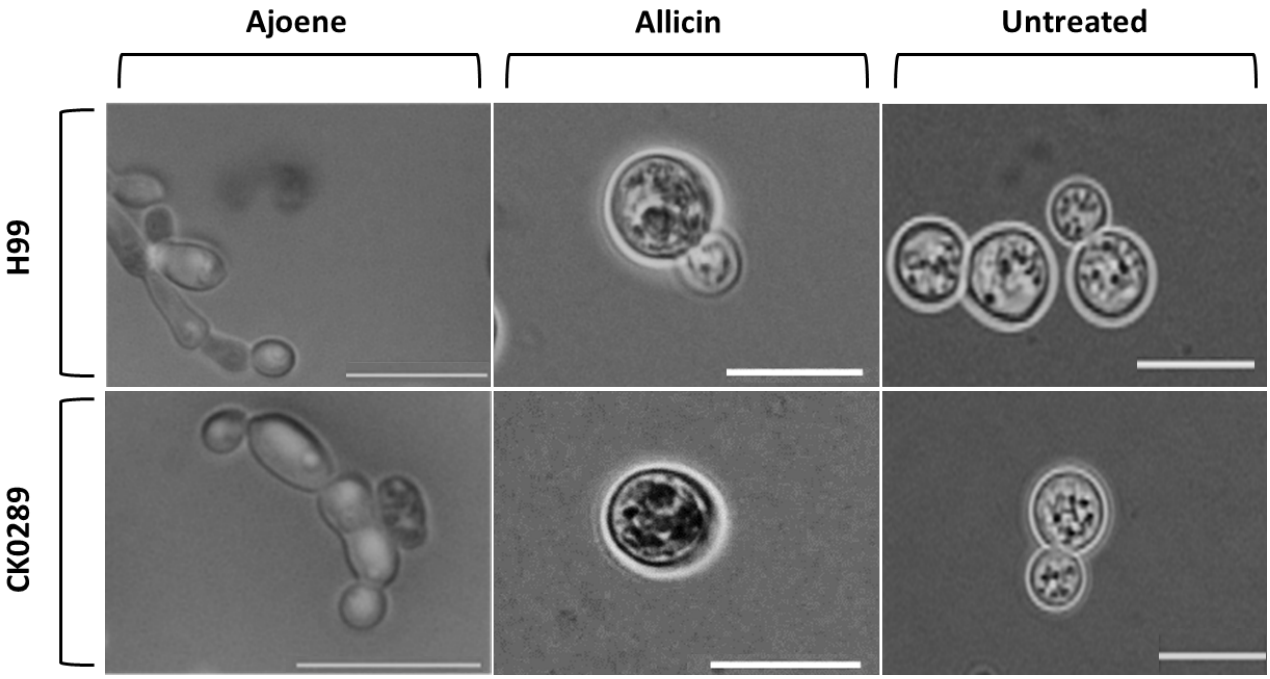


Figure 4.2: Light micrographs of *Cryptococcus neoformans* strains untreated or exposed to garlic organosulfur compounds. Strains represented by each row are shown on the left-hand side of the figure. Scale bars: 10 µm.

4.4.2.2 Natural garlic disulfides completely inhibited melanisation

Laccase activity was virtually abolished after *C. neoformans* H99 was exposed to allicin and ajoene (Fig. 4.3). The *C. neoformans* CK0289 QS mutant could not be tested for the inhibition of melanisation as the strain showed no laccase activity during each treatment (Chapter 3, Fig. 3.4). To test whether mQsp1 controls laccase activity during NL, the CK0289 QS mutant was exposed to synthetic mature Qsp1 (mQsp1). Exposing the QS mutant to mQsp1 should recovery its virulence phenotype to that of the H99 wild type strain. However, this was not the case. Because mQsp1 exposure did not affect laccase activity under NL, inhibition of melanisation by ajoene and allicin may be due to direct interference with factors involved in melanogenesis rather than QS.

Fungal laccase enzymes catalyse a one-electron redox reaction (Kunamneni et al., 2007): a substrate, such as L-DOPA, is radicalised by the addition of a single electron which can then undergo a cascade of spontaneous polymerisation events to form melanin particles. Ajoene and allicin may interfere with this process as they have previously been shown to exhibit antioxidant activity (Prasad et al., 1995; Naznin et al., 2010). Because ajoene and allicin appear to influence capsule thickness (Fig. 4.1) and melanisation (Fig. 4.3), important virulence factors required for host infection (Kwon-Chung et al., 1982; Zaragoza et al., 2009), the case for their potential as antivirulence agents against *C. neoformans* is strengthened.

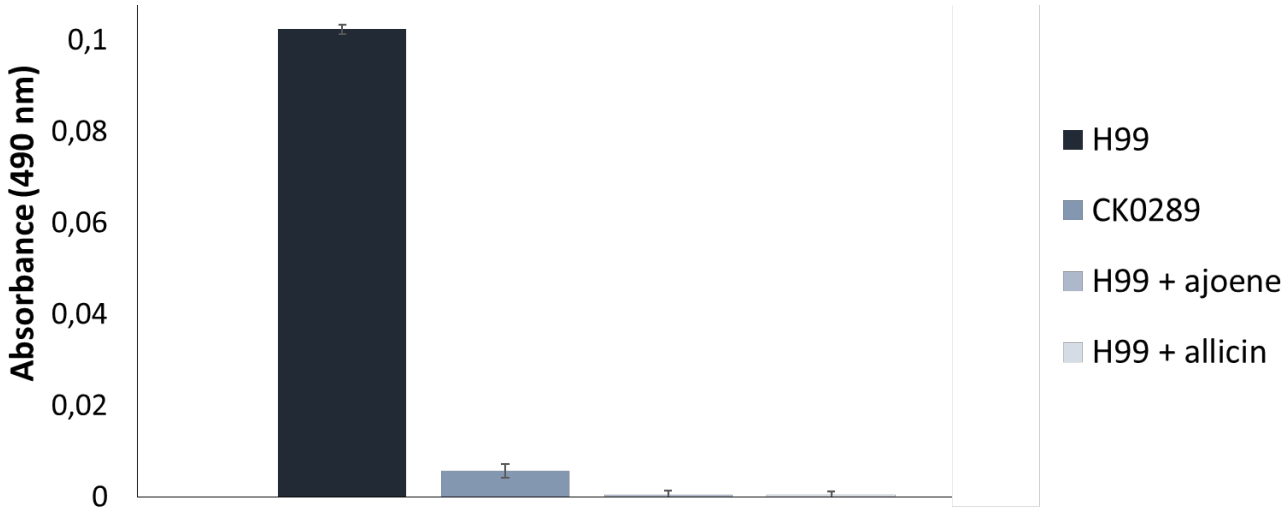


Figure 4.3: Melanisation of *Cryptococcus neoformans* strains grown in NLM-0.2 at 37°C for 48 h with or without exposure to 19 µg/mL ajoene and 13 µg/mL allicin. Melanisation was recorded as the absorbance of melanin intermediates produced via laccase activity at a wavelength of 490 nm. Error bars represent standard error of the mean (n = 3).

4.4.2.3 Natural organosulfides likely do not inhibit cryptococcal ureases

Urease activity for both *C. neoformans* strains exposed to allicin did not significantly differ from the untreated controls ($P > 0.05$) (Fig. 4.4). In contrast, Ranjibar-Omid and co-workers (2015) found that allicin inhibited the urease activity of the Gram-negative bacterium, *Proteus mirabilis*. Similar observations were made for the ureases of jack bean and ureases found in soil (Juszkiewicz et al., 2003; Mathialagan et al., 2017). Unexpectedly, ajoene exposure increased cryptococcal urease activity. The wild type strain showed a 2.3X increase in absorbance after ajoene treatment (0.386±0.001) relative to the untreated control (0.167±0.001) ($P < 0.001$), with similar but less pronounced observations made for the qsp1Δ mutant. Microscopic analyses previously showed that treatment with ajoene induced *C. neoformans* to switch to their hyphal state (Fig. 4.2). In filamentous form, fungal cells can scavenge nutrients more effectively, and their physiology also differs drastically from that of their yeast form (Gimeno et al., 1992). Therefore, the phenotypic switch induced after ajoene exposure, and the altered physiological and metabolic characteristics of cells in hyphal growth compared to yeast-like growth, may explain the pronounced urease activity observed.

Ureases are classified as sulfhydryl enzymes and are generally sensitive to compounds, specifically sulfurous compounds, which target sulfhydryl functional groups (Upadhyay, 2012). Ajoene and allicin are compounds that readily react with sulfhydryl groups, particularly those in cysteine residues (Winkler et al., 1992), which does not explain why urease activity remained unchanged or increased during organosulfide exposure (Fig. 4.4). The colourimetric assay used to measure urease activity in this study is sensitive to pH changes. Once the cell degrades urea and releases ammonia, the assay medium changes from yellow to pink depending on the amount of ammonia released (Cox et al., 2000; Olszewski et al., 2004). However, the major drawback to this assay is that it is non-specific and can thus be influenced by any substrate that modifies the local pH. This may explain the unexpected results measured in this study and, therefore, requires further investigation using an optimised, urease-specific assay.

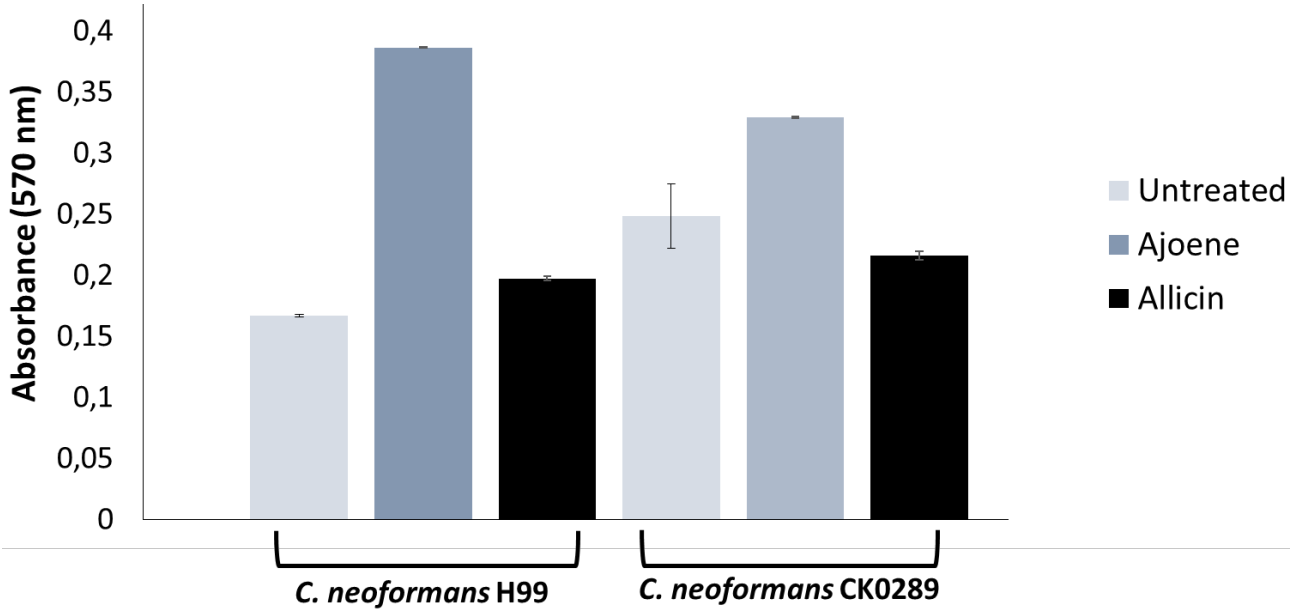


Figure 4.4: Urease activity for *Cryptococcus neoformans* strains exposed to 19 µg/mL ajoene and 13 µg/mL allicin during growth at 30°C for 48 h in nitrogen-limiting media containing 0.2 g/L NH_4Cl (NLM-0.2). Error bars represent the standard error of the mean (n = 3).

4.4.3 Allicin variably affects cell membrane ergosterol

Allicin did not inhibit cell membrane ergosterol content in both *C. neoformans* strains (Fig. 4.5). Instead, *C. neoformans* CK0289 showed a 1.3X increase in ergosterol content after exposure to allicin (0.0042±0.0001%) relative to the untreated control (0.0032±0.0002%) ($P = 0.02$). Treatment with FLC produced a 6X decrease in cell membrane ergosterol content measured for *C. neoformans* H99, as was expected ($P < 0.001$). The effect was similar for *C. neoformans* CK0289, but the decrease was less pronounced at 1.6X.

It was previously shown that inhibition of the high-osmolarity-glycerol (HOG) pathway, which regulates ergosterol biosynthesis, causes *C. neoformans* to become more resistant to FLC (Ko et

al., 2009). Because the CK0289 QS mutant appears to have a defect in ergosterol biosynthesis during growth in NLM and elevated temperature (Fig. 3.7 & Fig. 4.5), this observation may explain why it was less susceptible to FLC treatment than the wild type strain. These results are in contrast to the finding that the ergosterol content was increased in the CK0289 QS mutant after allicin treatment. Taken together, this implies that Qsp1 is not an absolute requirement for regulating ergosterol biosynthesis and that stress response factors independent of QS are involved. Due to the limited supply of non-commercial organosulfur compounds available for this study, the effect of ajoene on *C. neoformans* ergosterol content could not be tested.

Amphotericin B (AmB) and FLC are the main antifungal agents used to treat cryptococcal infections (Smith et al., 2015). These drugs respectively target the enzymes responsible for ergosterol biosynthesis and ergosterol itself (Holz, 1974; Bossche et al., 2009). As Qsp1 is necessary to induce ergosterol synthesis for membrane stability under nutrient and temperature stress (Chapter 3, section 3.4.3), QS inhibition may sensitise *Cryptococcus* cells to membrane sterol-targeting drugs. Therefore, QQ drugs could potentiate the activity of FLC and AmB and improve their efficacy in treatment programs.

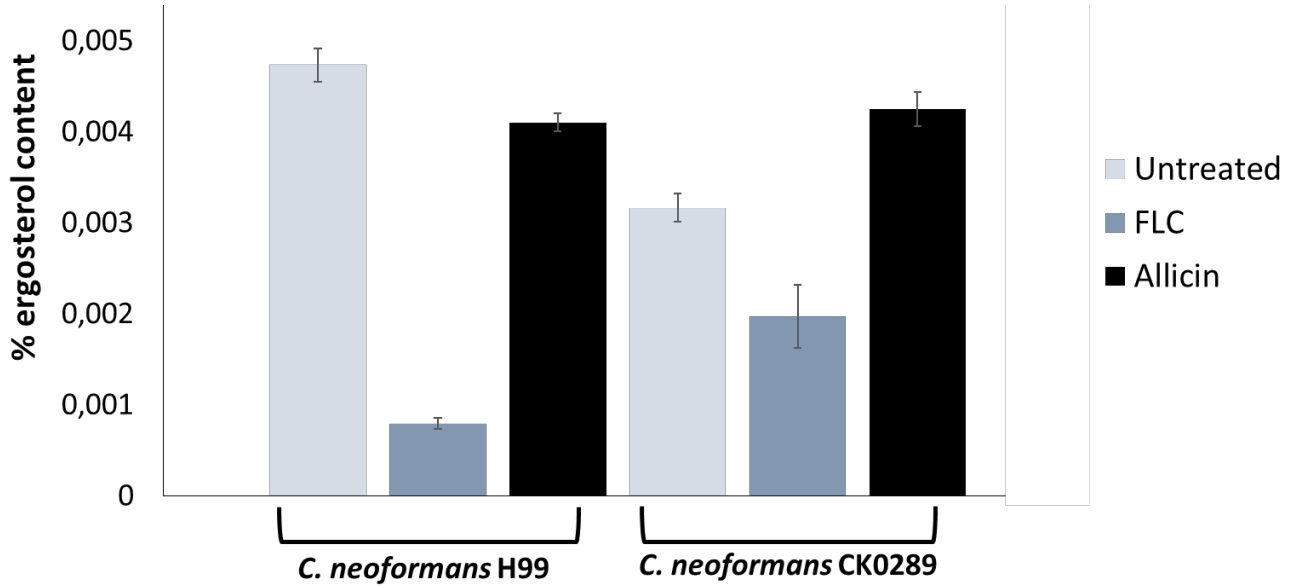


Figure 4.5: Ergosterol content as mean percentage wet pellet weight determined for *Cryptococcus neoformans* strains grown with or without different inhibitors in nitrogen-limiting media containing 0.2 g/L NH_4Cl (NLM-0.)2 at 37°C for 48 h. Error bars represent standard error of the mean (n = 3).

4.5 Conclusion

Mortality associated with *C. neoformans* infections and the lack of access to effective treatment in regions with the highest burden of cryptococcal meningitis highlights the need for the development of alternative solutions. Preliminary evidence for a subset of *C. neoformans* virulence factors in this study indicated that garlic-derived organosulfides are antivirulent and may act on multiple cellular

targets. In modern drug design, ideal drug candidates are those that have high specificity, target non-essential cellular components and have multiple targets as this reduces the chance of infectious organisms acquiring resistance to them (Csermely et al., 2005; Espinoza-Fonseca, 2006; Cegelski et al., 2008; LaSarre and Federle, 2013; Kirienko et al., 2019). However, investigating whether this theory holds for drugs that have non-essential targets, such as virulence factors or QS, is challenging since few known antipathogenic drugs have been approved for clinical use (Imperi et al., 2019).

Natural doses of ajoene, allicin and other organosulfides may be acquired by ingesting crushed, raw garlic. One garlic clove can weigh approximately 2-4 g (Lawson et al., 1998) and can produce about 2.5 mg of allicin (Slusarenko et al., 2008). Although the World Health Organisation recommends the consumption of 2-5 g of fresh garlic per day for effective results, in the literature this amount differs greatly and ranges between 900 mg to 10 g of garlic per day (Abdullah et al., 1989; Kandil et al., 1987; Kandil et al., 1988; Amagase et al., 2001; Tattelman, 2005). As this may not be practical and many people may not be in favour of consuming garlic daily, due to its taste and offensive odour, numerous methods have been developed to chemically synthesise garlic compounds (Nikolic et al., 2003; Persson et al., 2005; Hunter et al., 2008; Chen et al., 2015; Silva et al., 2018). The chemical synthesis of garlic compounds can help with standardising drug formulations, eliminating the variability in the composition and concentration of bioactive compounds in each garlic clove.

The garlic compounds, ajoene and allicin, are rapidly absorbed into the bloodstream when taken orally (Lin et al., 2015) and, together with other organosulfides, can even cross the blood-brain barrier (Miron et al., 2000). Therefore, these compounds could be administered cheaply without the need for intravenous catheters and may even target cryptococcal cells in brain tissue. Although these compounds will likely not replace mainstay anticryptococcal drugs, they are promising candidates for alternative cryptococcal therapy and may be beneficial in resource-poor settings where access to AmB and flucytosine is restricted.

In this study, the antipathogenic activity of ajoene and allicin were shown not to be due to the inhibition of QS. However, further studies are required to confirm this. An attempt to design a biosensor that can monitor *C. neoformans* QS responses was made (Chapter 5) with the application of screening the garlic organosulfides, and other potential compounds, for QQ activity. Gaining further insight into the factors involved in the QS response of *C. neoformans* could aid in the development of novel anticryptococcal drugs and treatment strategies.

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CHAPTER 5

RESEARCH RESULTS III

Engineering a whole cell-based *Cryptococcus neoformans* quorum sensing biosensor

Chapter 5: Engineering a whole cell-based *Cryptococcus neoformans* quorum sensing biosensor

5.1 Abstract

Living organisms have evolved specialised sensing machinery to monitor and respond to changes in their environment. With the advancement of molecular biology, various elements of biological sensing apparatus have been manipulated to design sensitive analytical devices called biosensors. Biosensors provide quantitative ways to investigate changes in systems and allow one to study how biological entities respond to these changes in real-time, with applications in medical diagnostics, drug discovery, food safety, process control and environmental monitoring. The causative agent of fungal meningitis, *Cryptococcus neoformans*, produces an 11-mer peptide (Qsp1) that regulates a quorum sensing (QS) network by binding directly to promoter regions of genes encoding virulence-associated transcription factors. We aimed to design a whole cell-based QS biosensor in *Saccharomyces cerevisiae* using these known Qsp1-regulated promoters fused to a green fluorescent protein reporter gene. The biosensor would be able to respond to extracellularly added synthetic Qsp1 (mQsp1) and allow one to screen a selection of compounds that can potentially interfere with cryptococcal QS. We found that all QS-responsive elements failed to produce fluorescent signals in *S. cerevisiae*. This was likely due to the yeast being unable to import mQsp1 or to initiate transcription from cryptococcal promoters. Attempts to engineer the QS biosensor in *C. neoformans* using a multitude of transformation techniques were unsuccessful. Future efforts will involve the use of biolistics to transform the biosensor elements into *C. neoformans*.

5.2 Introduction

The act of biosensing is universal to all cell types across all kingdoms of life. Single-celled and multicellular organisms can detect various environmental stimuli and respond by triggering protective mechanisms or changes in growth when confronted with various stress or nutrient signals, respectively. Because microbial systems have evolved sensitive ways to sense and respond to many stimuli, these elements have been manipulated for use in engineered biological tools called biosensors (Adeniran et al., 2015). Simply defined, a biosensor is a biological device composed of biological entities, such as live cells or enzymes, able to monitor specific changes in the immediate environment and generates a measurable signal in response to these changes (Su et al., 2011). One of the earliest reports on the design and application of a biosensor was in 1962, where a potentiometric enzyme electrode was adapted to monitor glucose concentrations in aqueous solutions (Clark and Lyons, 1962). Since then, biosensors have been designed with increased complexity for use in a plethora of applications relevant but not limited to the environment, biotechnology and medicine (Gui et al., 2017; Carpenter et al., 2018; Shi et al., 2018). The advantages of using biosensors are numerous. Monitoring the production, removal or presence of a specific analyte without the need for laborious chemical methods are some of the most commonly

exploited benefits to using biosensors (Adeniran et al., 2015; Eggeling et al., 2015). Among other applications, these approaches can be used to evaluate how an organism responds physiologically to certain conditions or to screen for substances that act on the biosensing elements.

Three main types of biosensors have been developed: those that rely on Förster resonance energy transfer (FRET), RNA switches or other transcriptional regulators (Zhang et al., 2015). Current FRET- and RNA switch-based biosensors have limited application for broad, high-throughput screening due to low signal output or because of complexities involved in engineering riboswitches (Eggeling et al., 2015). Thus, major advancements have been made on designing transcription-dependent biosensors, such as those focused on transcription factors (TFs) and gene promoters. For these types of biosensors, whole cells are typically used as a host for the biosensing elements, and the transcriptional regulators are usually coupled to a reporter gene not related to the system being investigated. These include reporter genes that can cause cells to fluoresce, luminesce, change colour, alter growth rate and even provide an electrical output in response to particular conditions (Adeniran et al., 2015). These signals are all measurable and can be quantitatively interpreted according to the biosensor's application. Although transcription-based responses can be measured using quantitative polymerase chain reaction (qPCR), cells first need to be lysed to collect short-lived RNA molecules and results are usually obtained hours after the initial biological response. On the other hand, biosensors can allow for the fast, sensitive and selective tracking of live biological signals both *in vivo* and *in vitro* (Daunert et al., 2000; Idil et al., 2017). This makes the use of whole-cell molecular-based biosensors more appealing and attests to their increased use in multiple fields of research.

The causative agent of cryptococcal meningitis, *Cryptococcus neoformans*, is an actively studied opportunistic pathogen of immune-compromised individuals (Perfect and Bicanic, 2015). *Cryptococcus* can overcome the attack by host immune effectors and survive the elevated temperature and low nutrient availability of the host due to the production of biosynthetic products that enhance the fitness of the pathogen. These biosynthetic products are often associated with disease development and progression and are thus called virulence factors (Casadevall and Pirofski, 2001; Wu et al., 2008). In most pathogenic fungal and bacterial organisms, the expression of virulence factors is regulated by a chemical communication system known as quorum sensing (QS). Small signalling molecules are released by the cell, which, once reaching a threshold concentration, are detected and processed to induce a QS response. This process causes cells to synchronise their genetic and metabolic processes, coordinating them on a population level in a cell density-dependent manner (Whiteley et al., 2017). For pathogenic organisms, QS responses usually lead to the increased production of virulence factors, social growth in the form of biofilms, morphological transitioning from yeast to hyphal state and, consequently, heightened protection against harsh stressors (Alhede et al., 2009; van Gennip et al., 2009; Chiang et al., 2013; Lyons and Kolter, 2015).

Quorum sensing-like peptide 1 (Qsp1), a small 11 amino acid long peptide, was recently identified as an important signalling molecule for *C. neoformans* (Lee et al., 2007; Homer et al., 2016). The peptide is first produced intracellularly as an immature propeptide before being released outside the cell where it is extracellularly modified by a cell surface-bound peptidase to form mature Qsp1. Once there are high cell numbers and enough Qsp1 accumulates outside the cell, the peptide is transported back into the cell via an oligopeptide transporter. This oligopeptide transporter was previously identified as an orthologue of the *Saccharomyces cerevisiae* high-affinity glutathione transporter (Opt1) (Osawa et al., 2006). Inside the cell, Qsp1 stimulates an increase in the production of more Qsp1 (autoregulation) and modulates virulence by binding to the promoters of three virulence-associated genes encoding the TFs Gat201, Gat204 and Liv3. Further enhancing this signalling network, each TF stimulates the production of Qsp1 by binding to the *QSP1* gene promoter and also binds and regulates each other's promoters. Few studies have investigated QS in *C. neoformans* and what role Qsp1 plays in modulating the pathogen's physiological changes in response to various conditions (Lee et al., 2009; Albuquerque et al., 2014; Homer et al., 2016; Tian et al., 2018; Trevijano-Contador et al., 2018; Camacho et al., 2019).

In this study, we took advantage of the characteristic of Qsp1 binding to and regulating the transcription of the *GAT201*, *GAT204* and *QSP1* promoters, and aimed to engineer a *C. neoformans* QS biosensor in *S. cerevisiae* and *C. neoformans* using these QS-responsive promoters.

5.3 Materials and methods

All reagents were supplied by Merck (Darmstadt, Germany) unless otherwise indicated.

5.3.1 Yeast and bacterial strains

A *C. neoformans var. grubii* H99 (serotype A) strain was kindly provided by Dr. Joseph Heitman (Duke University, Durham, NC), and a *C. neoformans* CK0289 QS mutant strain (qsp1Δ; H99 background) was a kind gift from Prof. Hiten Madhani (University of California, San Francisco, CA). The *S. cerevisiae* Y294 and *C. neoformans* CK0289 strains were used as hosts of the QS biosensor genetic constructs. *Escherichia coli* DH5α was used for the propagation of all vectors constructed. See Table 5.1 and Table A5.1 (in appendix) for more details of strains used and constructed in this study.

The *E. coli* DH5α strain was recovered from freeze-cultures made in 50% (v/v) glycerol and grown on Luria Bertani (LB) solid medium at 37°C for 24 h. Yeast strains were recovered from freeze-cultures made in 40% (v/v) glycerol and transferred onto yeast extract peptone dextrose (YPD) solid medium and incubated at 30°C for 3 days. Revived cultures were stored at 4°C and used for a maximum of one week before preparing a fresh culture.

Table 5.1: Strains used in this study

Strain	Genotype	Source
E. coli DH5α	F- endA1 glnV44 thi-1 recA1 relA1 gyrA96 deoR nupG purB20 φ80dlacZΔM15 Δ(lacZYA-argF)U169 hsdR17(rK–mK+) λ–	Sambrook et al., 1989
C. neoformans H99	Serotype A *MAT***α** wild type	Joseph Heitman (Duke University)
C. neoformans CK0289	Serotype A *MAT***α** qsp1Δ::natR	Hiten Madhani (Homer et al., 2016)
S. cerevisiae Y294	MAT**α** leu2-3 leu2-112 ura3-52 his3Δ trp1 GAL^+ [cir^+]	ATCC 201160

5.3.2 Media and reagents

The YPD medium was prepared with 20 g/L peptone, 10 g/L yeast extract and 20 g/L glucose. Synthetic complete medium without uracil (SC^{-URA}) was prepared using 1.7 g/L yeast nitrogen base without amino acids and ammonium sulphate (BD Life Sciences, New Jersey, USA), 5 g/L ammonium sulphate, 20 g/L glucose, 2 g/L amino acid mix lacking uracil and adjusted to pH 6.0 with NaOH. For fluorescence assays, SCx medium was prepared in the same way as SC^{-URA} above, however, the concentrations of each reagent were doubled, the media was buffered with 20 g/L succinic acid and filter-sterilised with low protein binding 0.2 µm Pall Acrodisc PF syringe filters. The LB broth was prepared with 10 g/L tryptone, 5 g/L yeast extract and 10 g/L NaCl. The pH was adjusted to 7.5 with NaOH. All solid media contained 15 g/L agar. A HEPES/NaCl solution was prepared by mixing 10 mM HEPES (buffered to pH 7.4 with NaOH) and 100 mM NaCl (Shiba et al., 2017).

5.3.3 Biosensor construction

5.3.3.1 Gene promoter amplification and cloning

Primers were designed to recognise the promoter regions of known Qsp1-regulated genes in the *C. neoformans* genome (Homer et al., 2016; see Table 5.2 for primers and selected promoters). Promoters of *C. neoformans* were identified using the Ensembl Fungi database (https://fungi.ensembl.org/index.html). Identified *C. neoformans* QS promoters were directly PCR-amplified from *C. neoformans* H99 genomic DNA (gDNA) with Q5 High-Fidelity DNA Polymerase (NEB, Massachusetts, USA), using the manufacturer's thermocycling conditions. These PCR fragments were subsequently cloned into an *Eco*RV (Roche, Basel, Switzerland) blunt-ended pJET2.1 vector using the CloneJET PCR Cloning kit (ThermoFisher Scientific, Massachusetts, USA) and transformed into chemically competent *E. coli* DH5α cells.

All primers used for *C. neoformans* promoter amplification were designed to build in a terminal 5'-*Eco*RI and 3'-*Pac*I during PCR. The QS promoters were thus excised from pJET2.1 using *Eco*RI and *Pac*I restriction enzymes (NEB, Massachusetts, USA) followed by sub-cloning into the *Eco*RI

and *Pac*I sites of an intermediary pHK212 centromeric vector (Fig. A5.1 in appendix) to create the biosensor cassette (QS promoter-*EGFP-CYC1* terminator) (Fig. 5.1). Each biosensor cassette was then excised with *Hind*III and *Bcu*I restriction enzymes (ThermoFisher Scientific, Massachusetts, USA) and sub-cloned into two yeast integrative vectors, namely, YIplac211 (Gietz and Sugino, 1988) and pSDMA58 (Arras et al., 2015). See Table A5.1 in the appendix for details of the constructed vectors. YIplac211 and pSDMA58 constructs were used for *S. cerevisiae* and *C. neoformans* transformations, respectively. Positive control strains contained the *PGK1* promoter taken directly from the original pHK212 vector (Fig. A5.1 in appendix) or the *C. neoformans GAL7* promoter in the biosensor cassette.

Table 5.2: Primers targeting *C. neoformans* H99 QS-responsive promoters for PCR

Promoter	Gene Accession Number	Primer Pair	PCR Annealing and Extension Temperature (°C)	Amplicon Size (bp)
GAT201	CNAG_01551	CC**TTAATTAA**GAGGGACATCAACGAGAGGGATAGC	65	744
		C**GAATTC**GCCTAACAACCCTTCTAACCGTTC		
GAT204	CNAG_06762	C**GAATTC**GATTTGGGGTCACGTTCTCTCC	65	693
		CC**TTAATTAA**GGGTTACCAATTCCCATGCAAGTGG		
QSP1	CNAG_03012	CC**TTAATTAA**GGCCGTCTATCTCCTCCCGTATTC	65	797
		C**GAATTC**GAACGACAAGCACCCAACAC		

Areas of the primer sequences annotated with bold text indicate terminal *Eco*RI or *Pac*I (underlined) sites

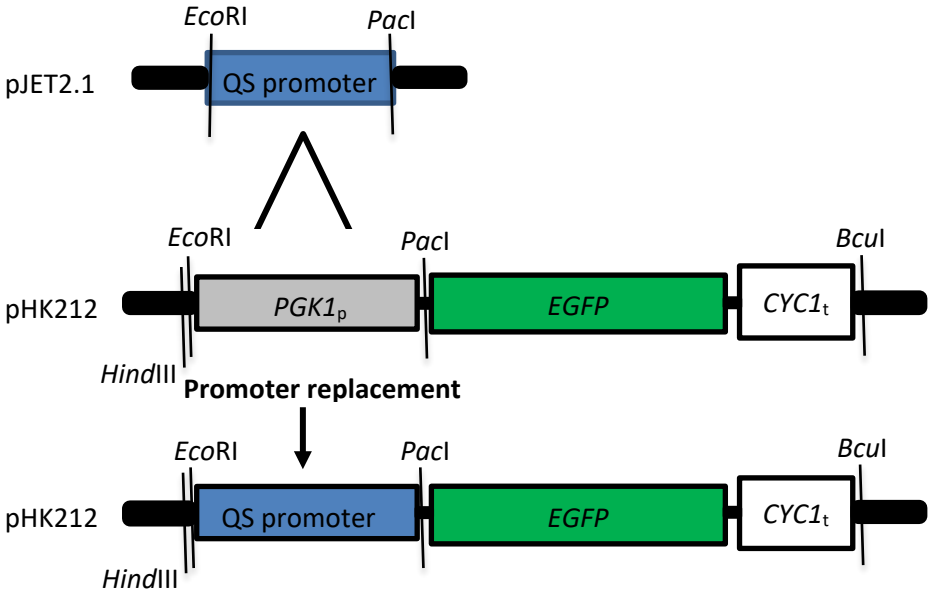

Figure 5.1: Diagram illustrating the strategy used to sub-clone *Cryptococcus neoformans* promoters from the initial pJET2.1 vector, after restriction digests with *Eco*RI and *Pac*I, into the intermediary pHK212 vector to create the quorum sensing biosensor cassette. Only relevant sections of the double-stranded circular plasmids are represented in the diagram.

5.3.3.2 Linearisation of plasmids for integration

YIplac211 and pSDMA58 final plasmids (Table A5.1 in the appendix, vectors 1-9) were linearised for integration into the genomes of the respective yeasts. YIplac211-based vectors were linearised within the *URA3* auxotrophic marker using *Stu*I (ThermoFisher Scientific, Massachusetts, USA). Inverse PCR with Q5 High-Fidelity DNA Polymerase was used to linearise pSDMA58-based vectors using primers designed to target the Safe Haven linker site on the vector (Table 5.3).

Table 5.3: Primer pair used to amplify pSDMA58-based vectors from the Safe Haven linker site

Primer Pair	PCR Annealing and Extension Temperature (°C)	Amplicon Size (bp)
CCTTAATTAAGAGGGACATCAACGAGAGGGATAGC	62	>8000 bp
CGAATTCGCCTAACAACCCTTCTAACCGTTC		

5.3.3.3 Yeast-mediated ligation

Yeast-mediated ligation was used to construct the *C. neoformans GAL7*-containing pSDMA58 (pSD-*GAL7-EGFP*) and YIplac211 (YIp-*GAL7-EGFP*) vectors. A forward primer (GAL7FWD_pHK) was designed with a 50 bp flanking region homologous to pHK212 and 20 bp homology to the *GAL7* promoter. A reverse primer (GAL7REV_EGFP) was designed with a 50 bp flanking region to the 5'-end of the *EGFP* gene in pHK212 and had 19 bp homology to the *GAL7* promoter region (Table 5.4). These primers were used to isolate the 800 bp promoter region upstream of the *GAL7* gene (CNAG_06052) from the *C. neoformans* H99 genome via PCR.

Table 5.4: Primers used for the homologous recombination of the *GAL7*-containing biosensor cassette

Primer	Sequence	PCR Annealing and Extension Temperature (°C)
GAL7FWD_pHK	**CGGGCCCCCCCTCGAGGTCGACGGTATCGATAAGCTTGATAT CGAATTCG**GTCAGACAGGTGAGATACTC	74
GAL7REV_**EGFP**	GGAGGACTTGCGGCTAATAAAGATGTAAATGAAACCATTTAATT AAGG**GGAATTTATGTTGAACGGG**	

Areas of the primer sequences annotated with bold text indicate sites with homology to the pHK212 vector (underlined) and the area with homology to the *GAL7* promoter

The pHK212 vector containing the *GAT201* promoter (pHK-*GAT201-EGFP*) was prepared for homologous recombination by excising the promoter region of the biosensor cassette using *Pac*I and *Hind*III restriction enzymes. This linear, promoter-less vector and the *GAL7* PCR-amplified fragments were co-transformed into *S. cerevisiae* Y294 and selected on SC^{-URA} plates. The pHK212 vector containing the *GAL7-EGFP-CYC*1 cassette (pHK-*GAL7-EGFP*) was isolated from transformants using a method described by Hoffman and Winston (1987) and transformed into

chemically competent *E. coli* DH5α cells. Thereafter, the *GAL7-EGFP-CYC1* cassette was excised using *Hind*III and *Bcu*I restriction enzymes and sub-cloned into YIplac211 (YIp-*GAL7-EGFP*) and pSDMA58 (pSD-*GAL7-EGFP*). The YIp-*GAL7-EGFP* and pSD-*GAL7-EGFP* vectors were transformed into *S. cerevisiae* Y294 and *C. neoformans* CK0289, respectively.

5.3.4 *Saccharomyces* transformation

Saccharomyces was transformed using the lithium acetate (LiAc) method as described by Gietz et al. (1995). Transformed cells were spread-plated onto solid SC^{-URA} plates and incubated for 3 days at 30°C. Colonies observed after incubation were re-streaked onto fresh SC^{-URA} plates and screened for the presence of the integrated vectors using colony PCR.

5.3.5 *Cryptococcus* transformation

5.3.5.1 Lithium acetate

The same LiAc method as described above for *S. cerevisiae* transformations was used; however, *C. neoformans* transformants were selected on YPD medium supplemented with 150 µg/mL Hygromycin B (Invitrogen, California, USA).

5.3.5.2 Electroporation

Three electroporation protocols were used as described by Cox et al. (1996), Masaki et al. (2012) and Lin et al. (2015), with slight modifications to the electroporator settings. For the Cox et al. protocol, electroporator settings were adjusted to a voltage of 0.49 kV, capacitance of 25 or 50 µF and resistance of 360 or 400 Ω. No changes were made to the electroporator settings described by the other protocols. Electroporation was performed using the Gemini BV Gene Pulser Electroporation system (Bio-Rad Laboratories, California, USA). Concentrations of approximately 0.5-10 µg DNA were used for transformations and transformants were selected on YPD medium supplemented with 150 µg/mL Hygromycin B (Invitrogen, California, USA).

A fourth method was used, which included an additional capsule reduction step to each of the above electroporation protocols as described by Bryan et al. (2005). Briefly, *C. neoformans* cells were prepared using the electroporation methods mentioned above. However, after initial harvesting, cells were washed twice with 40% (v/v) dimethylsulfoxide (DMSO). Before each wash step, cells were incubated in DMSO for 30 min at room temperature. Following this, cells were harvested and the same electroporation protocols described above were followed. Cells were plated out onto YPD medium without antibiotic after DMSO wash steps to check for viability.

5.3.5.3 Spheroplasting

Spheroplasts of *C. neoformans* were prepared using a method as described by Varma and Kwon-Chung (1991), followed by a standard spheroplast transformation protocol (Becker and

Lundblad, 2001). Spheroplasting solution contained SCS buffer (20 mM sodium citrate buffer at pH 8.0, 1 M sorbitol) with 50 mg/mL Novozyme 234 lysing enzyme (*Trichoderma harzanium*), in which cells were incubated for 1 h at 37°C on a rotating wheel. Transformants were selected on YPD medium supplemented with 1 M sorbitol and 80-150 µg/mL Hygromycin B (Invitrogen, California, USA)). The plates were incubated at 30°C for 2-3 days. Spheroplasts were plated out onto YPD medium containing 1 M sorbitol without selection before and after transformation steps to check for viability.

5.3.6 Fluorescence induction

For the induction of the QS-responsive promoters in the constructed biosensors, a chemically synthesised mature Qsp1 (mQsp1) (GL Biochem (Shanghai) Ltd., Shanghai, China) was used. Cells were cultured for 24 h in 5 mL SCx medium at 30°C with shaking. Cells were harvested and grown for an additional 3 h in fresh SCx medium containing 33 µM mQsp1 at 30°C (Lee et al., 2007). Strains containing the *GAL7* promoter were incubated in SCx medium containing 2% (m/v) galactose instead of glucose. After incubation, cells were harvested, resuspended in Tris-EDTA buffer (pH 6.0) and fluorescence detected using fluorescence microscopy (Nikon Eclipse E400 fluorescence microscope, Laboratory Optical Service, Inc., USA) with the 480/30 FITC excitation filter.

5.3.7 Morphology recovery and peptide import assays

The functionality of mQsp1 was assessed by using a morphology recovery assay as described by Homer et al. (2016), with slight modifications. Briefly, a *C. neoformans* CK0289 culture grown for 24 h in YPD broth at 30°C was harvested, washed and serially diluted in PBS from 10^{-1} to 10^{-10}. A 140 µL volume of the 10^{-10} dilution of cells was mixed with 10 µL 33 µM mQsp1 and spread onto solid YPD medium using sterile 5 mm glass beads. The plates were incubated at 30°C for 3 days whereafter colony morphology was observed.

To determine whether mQsp1 can be transported into *S. cerevisiae,* a synthesised mQsp1 N-terminally conjugated to the fluorescent label, 5-TAMRA (5-carboxytetramethylrhodamine) (GL Biochem (Shanghai) Ltd., Shanghai, China), was used. The fluorescent-labelled peptide (TAMRA-mQsp1) was dissolved in DMSO and stored at -20°C in the dark. Yeast cultures grown in 5 mL YPD broth for 24 h at 30°C were harvested and resuspended in HEPES/NaCl solution to a cell density of 1 x 10^6 cells/mL. To this, 33 µM TAMRA-mQsp1 was added and the cultures were incubated at 30°C for a further 30 min to 1 h in the dark. Samples from these peptide-exposed cultures were washed, resuspended in HEPES/NaCl solution and then visualised using a fluorescence microscope (Nikon Eclipse E400 fluorescence microscope, Laboratory Optical Service, Inc., USA) with the 560/40 Texas Red excitation filter. As positive controls, *C. neoformans* H99 and *C. neoformans* CK0289 samples were included. Negative controls included yeast cells without peptide exposure. TAMRA-mQsp1 was also used in the colony phenotype recovery assay as performed for mQsp1.

5.4 Results and discussion

5.4.1 Biosensor construction in *S. cerevisiae*

The well-studied yeast, *S. cerevisiae*, was chosen as a favourable host for the QS biosensor as it is readily transformable, genetically robust and will allow one to safely and more easily investigate facets of the cryptococcal QS response without the risk of pathogen exposure. It would therefore also be much easier to replace and add different elements to the biosensor to widen the scope of its application. The transformation of all final biosensor constructs in *S. cerevisiae* were successful, generating the biosensor strains 10-14 (Table A5.1 in appendix). However, all strains containing cryptococcal promoters failed to produce a measurable fluorescent signal after exposure to mQsp1 (data not shown). The strain containing the *GAL7* promoter also failed to produce a measurable signal after galactose induction. Only the positive control strain containing the constitutive *S. cerevisiae PGK1* promoter (Y294_YIp-PGK-*EGFP*) fluoresced (Fig. A5.2 in appendix), confirming that the reporter protein was functionally expressed. The lack of a fluorescent signal detected for the biosensor constructs containing the cryptococcal promoters suggests four possibilities: (1) *Saccharomyces* lacks the transcriptional machinery necessary for recognising *Cryptococcus* promoters. (2) The *S. cerevisiae* OPT1 (scOPT1) protein may not be able to transport mQsp1 into the cell. (3) The synthetic QS peptide could be non-functional. (4) In addition to mQsp1, other factors produced explicitly by *C. neoformans* may be necessary to induce an optimal QS response.

5.4.1.1 Recognition of *Cryptococcus* promoters by *S. cerevisiae*

Two of the promoters, *GAT201* and *GAT204*, used in the construction of the QS biosensor are those that encode for TFs that control the expression of virulence genes in the basidiomycete, *C. neoformans*. As previously mentioned, these virulence-associated TFs, together with Liv3, bind to each other's gene promoters and to that of *QSP1* (Homer et al., 2016). They are important for enhancing the cryptococcal QS response by influencing transcription at these promoters. The TFs, Gat201, Gat204 and Liv3, are all absent in the ascomycete, *S. cerevisiae*, and no orthologues of them exist for this yeast. Many different TFs can bind similar *cis*-regulatory elements found within promoters, and a single promoter may even contain multiple binding sites for the same TF species (Ezer et al., 2014). This suggests that TFs can potentially work interchangeably with one another to initiate transcription. Therefore, *Saccharomyces* may possess a group of alternative TFs that can bind to and regulate the expression of *Cryptococcus* promoters. The TF binding site (TFBS) prediction tool, YEASTRACT (YEAst Search for Transcriptional Regulators And Consensus Tracking; http://www.yeastract.com) (Miguel et al., 2006; Miguel et al., 2018), was used to investigate this. Each of the *C. neoformans* QS-related promoters appears to harbour multiple potential *S. cerevisiae* TFBSs with a range of different TFs capable of recognising these sites (Table 5.5). Despite this, it is possible that Gat201, Gat204 and Liv3 are still necessary to promote the binding of mQsp1 to these sites and, in turn, combinatorially recruit necessary transcriptional machinery, or form part of the transcriptional complex to initiate transcription from these promoters. Their absence

in *S. cerevisiae* could, therefore, explain why no fluorescent output was detected after the biosensor was exposed to the QS peptide.

Table 5.5: List of predicted numbers of transcription factor binding sites (TFBSs) on *Cryptococcus neoformans* quorum sensing promoters recognised by *Saccharomyces cerevisiae* transcription factors (TFs)

Promoter	No. of TFBSs	No. of different *S. cerevisiae* TFs capable of binding
GAT201	68	32
GAT204	50	24
QSP1	65	28
GAL7	59	31

Although unlikely, it is possible that the length of some of the promoters selected was too short and thus *cis*-regulatory elements essential for the recognition of these promoters were missing. However, a functional *GAL7* promoter was characterised to be 650 bp in length (Wickes and Edman, 1995) and most yeast and other eukaryotic promoters are defined as being 455-1000 bp long (Haverty et al., 2004; Patil et al., 2004; Kristiansson et al., 2009). All promoters used in this study ranged between 690-800 bp (Table 5.2), falling within the array of reported fungal promoters. Overall, hardly any literature shows an ascomycete expressing a gene using a basidiomycete-derived promoter. Usually, when employing an ascomycetous yeast for the heterologous expression of a basidiomycete gene, the promoters used are native or originate from another ascomycete (López et al., 2016). This same approach holds for most other fungal and bacterial systems that rely on transcriptional regulators, especially biosensors (Eggeling et al., 2015). In light of this, it may be more favourable to engineer a *C. neoformans* QS biosensor in a basidiomycete. Unfortunately, there is a lack of developed genetic systems for basidiomycetous yeasts. Most basidiomycetous model systems that can easily be genetically manipulated are pathogens or mushrooms (Baronian, 2004; Rekengalt et al., 2007; Lin et al., 2008; Sugano et al., 2017). Transforming *S. cerevisiae* with a gene cassette that encodes for the production of the Gat201, Gat204 and Liv3 TFs (singularly or in combination) could also possibly help the yeast recognise the QS-responsive cryptococcal promoters and initiate transcription. Combining this TF-producing construct with the biosensor cassette used in this study would create a synthetic regulatory network mimicking the autoinducing system native to *C. neoformans*. Theoretically, this autoinductive network can be induced after mQsp1 exposure, enabling one to monitor *Cryptococcus* QS together with known key regulators of this process.

5.4.1.2 Comparison between *S. cerevisiae* OPT1 and *C. neoformans* OPT1

The oligopeptide transporters, scOPT1 and *C. neoformans* OPT1 (cnOPT1), are both high-affinity glutathione transporters also capable of transporting other small oligopeptides (Osawa et al., 2006).

These transporters are orthologous and have no significant differences in protein size, with scOPT1 being 799 amino acids in length and cnOPT1 being 791 amino acids long. However, when looking closely at their protein sequences, they have a sequence identity and similarity of approximately 42% and 61.8%, respectively. It could be likely that these transporters have different tertiary structures and thus have different peptide transport capabilities. Literature supports scOPT1 as a transporter of peptides that are generally 4-5 amino acids long (Miyake et al., 1998; Bourbouloux et al., 2000). No evidence exists to support scOPT1 having the capacity to mediate peptides of larger sizes, supporting the possibility that it may not be able to facilitate the import of the 11-mer *C. neoformans* QS peptide. No studies have investigated the plasticity of the *C. neoformans* OPT1p in conveying different sizes of peptides, but its capacity to transport mQsp1 suggests that it could potentially transport other peptides larger than 4-5 amino acids in size. If one can track the movement of the QS peptide to determine whether it can enter the *Saccharomyces* cell and it does, this would suggest that the *Cryptococcus* promoters are certainly not being recognised and transcribed. Because the QS biosensor constructed in this study relies on the extracellular addition of mQsp1 for promoter induction and if mQsp1 does not enter the cell, it might be necessary to express the cnOpt1 in *S. cerevisiae* to allow for proper uptake of the QS peptide. Another solution to ensure that *S. cerevisiae* can gain access to mQsp1 is to engineer the yeast to express the mature form of the QS peptide intracellularly. In this way, the yeast cell bypasses the requirement to import the peptide and removes the challenges associated with expressing a functional non-native transmembrane protein. An additional advantage is that there would be no requirement to extracellularly add the autoinducing peptide to the system, making the system more robust and cost-effective. The major drawback of this approach is that the production and absolute concentration of mQsp1 would not necessarily be controlled. However, under optimised conditions, the system would still allow for the monitoring of a change in the fluorescent signal when exposed to different substances. Nonetheless, the evidence does lean more toward *Saccharomyces*' inability to initiate transcription from *C. neoformans* promoters as opposed to a failure to import mQsp1 because the *GAL7-EGFP-CYC1* construct, which does not rely on mQsp1 import but instead on galactose induction, did not produce a fluorescent signal (data not shown).

5.4.1.3 The functionality of the synthetic quorum sensing peptides

To confirm if mQsp1 can enter *Saccharomyces* via scOPT1 and whether the failure to import resulted in no observed fluorescent signals for the QS-responsive promoters, a fluorescent-tagged mQsp1 (TAMRA-mQsp1) was used to track its movement into the cell. Fluorescence was observed for a few cells for all yeast strains tested (Fig. 5.2; blocks A and B). However, comparing the number of fluorescent cells to the total number of cells visible during microscopic analyses placed doubt on whether the peptide was being imported by live cells, especially since the *C. neoformans* strains should naturally take up this peptide. The fluorescing cells could likely be dead with disrupted cell membranes, allowing TAMRA-mQsp1 to permeate the cells more readily and non-specifically. This

was confirmed after staining and visualising heat-killed cells (95°C for 30 min), where all cells in view were fluorescent (Fig. 5.2; blocks C and D). The bulky 5-TAMRA fluorophore attached to mQsp1 likely interferes with the interaction between the peptide and the Opt1 transporter, preventing it from entering the cell. For *C. neoformans*, the fluorescent peptide could become lodged in the outer layers of the polysaccharide capsule, as it first needs to pass through this thick, mesh-like polymer before reaching the cell membrane. Since we could not track the movement of Qsp1 into *Saccharomyces*, it was not possible to confirm whether the biosensor failed to provide a fluorescent signal due to the peptide not being transported into the cell or whether the *Cryptococcus* promoters are not being transcriptionally recognised.

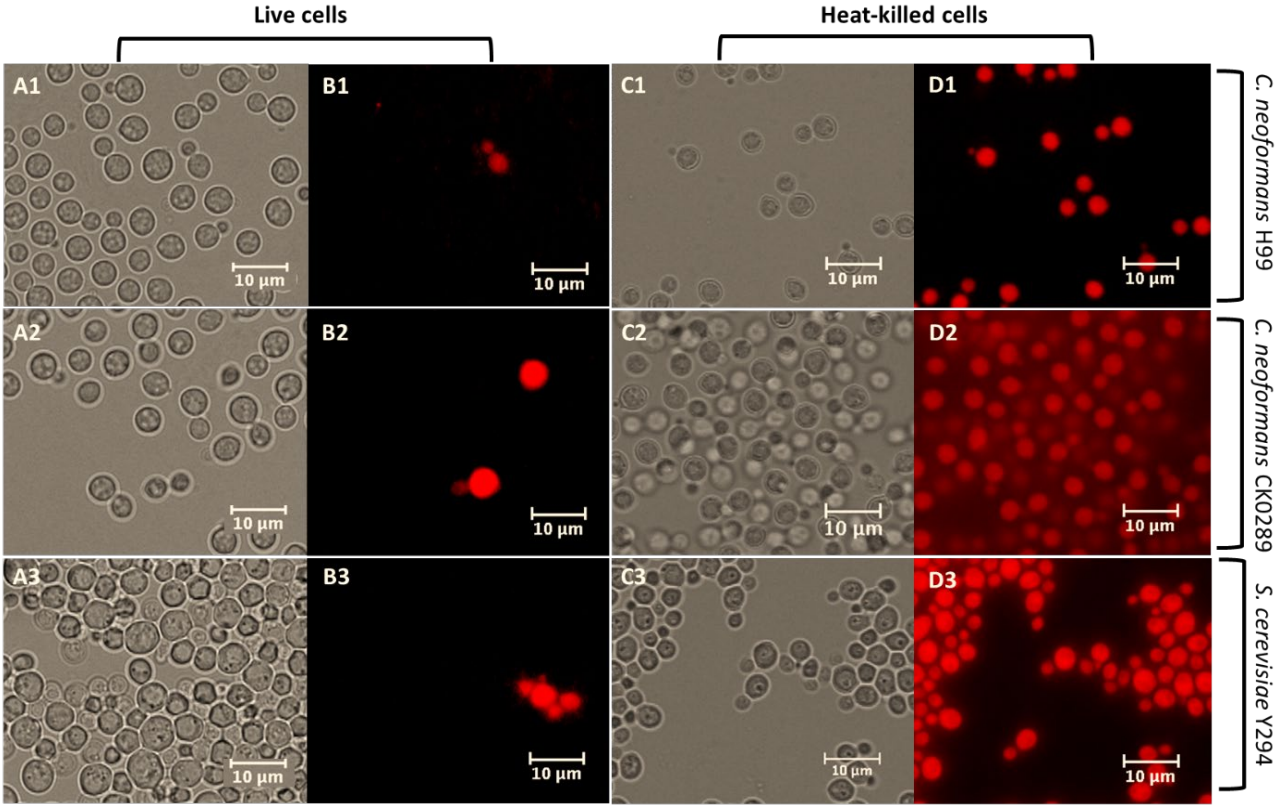

Figure 5.2: Fluorescent micrographs of yeast cells exposed to the fluorescent-tagged synthetic mature Qsp1 (TAMRA-mQsp1). Blocks A and C indicate live and heat-killed cells, respectively, for each yeast visualised under visible light. Blocks B and D indicate fluorescent images of rows A and C, respectively. Red colour illustrates the intracellular accumulation of TAMRA-mQsp1. Strains represented by each row are shown on the right-hand side of the figure. Scale bars: 10 µm.

Because the fluorescent peptide did not appear to be entering even the native *Cryptococcus* producer, this incited concern as to whether the non-fluorescently labelled mQsp1 is functional. A phenotype recovery assay was used to investigate this. The assay relies on the noticeable change from the dry and wrinkled morphology of the *C. neoformans* CK0289 QS mutant colonies to that of the smooth and mucoid colonies of the wild type strain (Homer et al., 2016). After exposure, mQsp1 lacking the fluorescent tag recovered the colony phenotype of the qsp1Δ mutant (Fig. 5.3C). This suggests that the non-labelled synthetic peptide was successfully transported into the metabolically

active cryptococcal cells and is likely functional. In contrast, TAMRA-mQsp1 failed to recover the CK0289 QS mutant's colony phenotype (Fig. 5.3D), confirming what was observed during fluorescent microscopic analyses: the fluorescent-labelled peptide was not entering live cells. Therefore, the hypothesis that the bulky 5-TAMRA tag inhibits the proper passage of the peptide into the cell is further supported.

Overall, no definitive proof was obtained to suggest that mQsp1 could enter *Saccharomyces*. Therefore, it cannot be confirmed whether the QS biosensor designed in this yeast failed at the point of peptide import or promoter recognition. Nevertheless, we do know that synthesised mQsp1 appears to be functional and could still possibly be used in combination with the *C. neoformans* CK0289 QS mutant to study various QS-related responses on a phenotypic level.

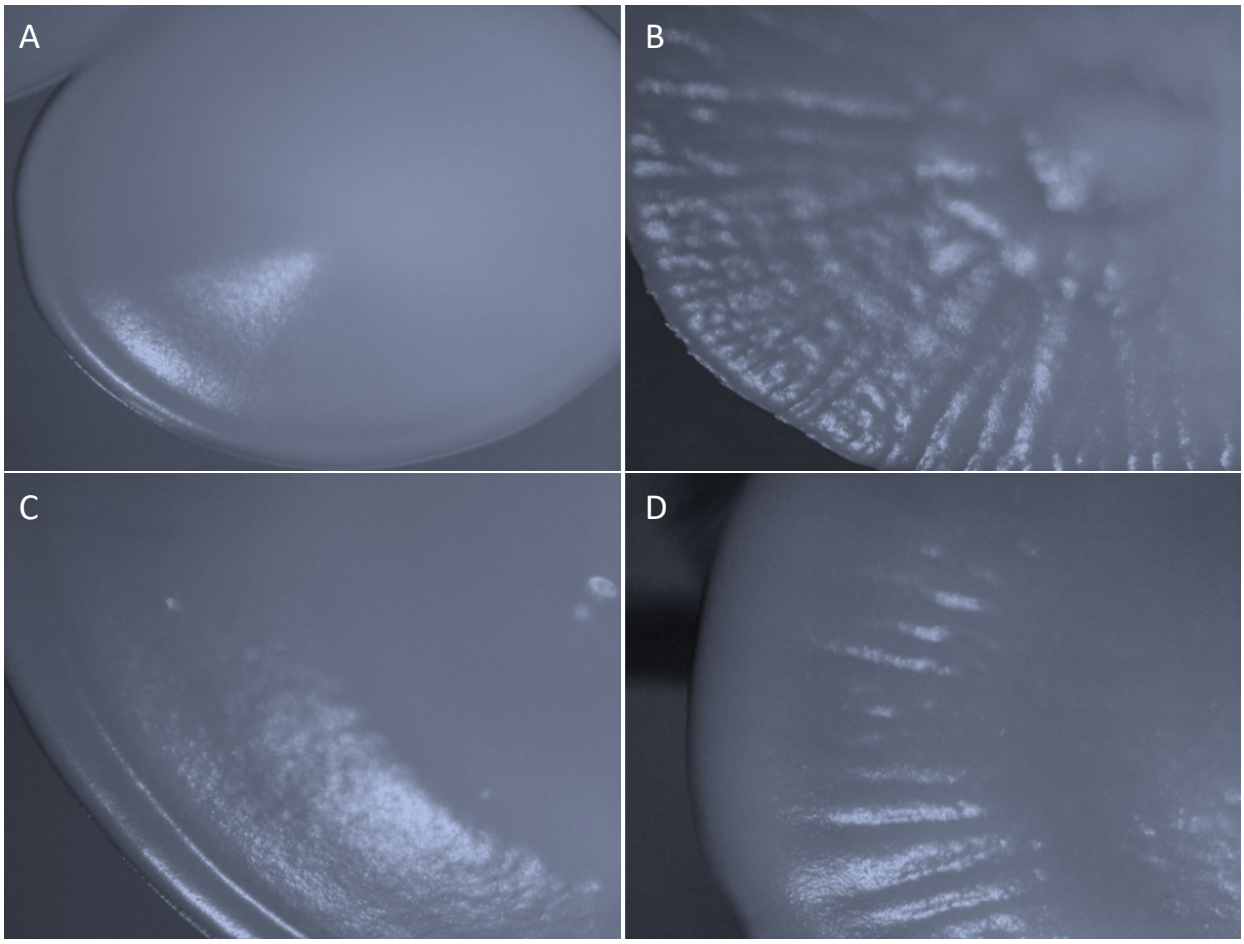

Figure 5.3: Macrographs displaying the morphology of *Cryptococcus neoformans* colonies. (A) Smooth *C. neoformans* H99 and (B) wrinkled *C. neoformans* CK0289 (qsp1Δ) colonies. (C) and (D) show *C. neoformans* CK0289 colonies after exposure to synthetic mature Qsp1 (mQsp1) and fluorescent-tagged mQsp1 (TAMRA-mQsp1), respectively.

5.4.2 Transformation of *C. neoformans*

Owing to the unsuccessful construction of the QS biosensor in *S. cerevisiae,* an attempt to construct the biosensor in *C. neoformans* was made as it is more likely to import mQsp1 and recognise its own

promoters. Extrachromosomal DNA, such as episomal plasmids, cannot be stably maintained by *C. neoformans* (Arras et al., 2015). Therefore, the genetic material must be integrated into the genome of the yeast if long-term maintenance and recombinant gene expression are desired. Integration would result in single-copy gene expression, which would make a biosensor capable of detecting signals more sensitively and with higher specificity (Knusden et al., 2014). Hence the reason integrative pSDMA58 and YIplac211 vectors were chosen for the biosensors constructed in this study. Another factor to consider in the design of the QS biosensor in *C. neoformans* is the inducer of the reporter signal. Because induction of QS with mQsp1 would be used to screen for promoter activity, the risk of background interference during signal detection is high due to native Qsp1 production in the wild type strain. It was therefore decided that the *C. neoformans* CK0289 QS mutant be used as the biosensor host. This mutant strain is incapable of producing its own Qsp1 but is still able to recognise and import the QS peptide from the environment (Homer et al., 2016). This would ensure that, during all experimental procedures, the time of exposure and concentration of mQsp1 is controlled to allow for accurate and valid comparisons between samples and between treatments.

Unfortunately, no positive transformants for *C. neoformans* were obtained after LiAc and all electroporation treatments tested. It was not surprising that LiAc was ineffective because only one study previously reported positive results when chemically transforming this yeast (Ou et al., 2011). Previously, transforming *Cryptococcus* via electroporation has been successful (Edman and Kwon-Chung, 1990; Varma et al., 1992; Lin et al., 2015). However, this technique fails to deliver DNA efficiently into the nucleus of the yeast (Davidson et al., 2000). It has been noted that the ability to transform *C. neoformans* via electroporation is strain-dependent as serotype D strains are more amenable to this process than serotype A strains (Lin et al., 2015), which were used in this study. DNA must cross multiple barriers before it can enter the cryptococcal cell. These barriers include a thick, multi-layered polysaccharide capsule, the cell wall and a layer of melanin aggregates (Camacho et al., 2019). Because these barriers may affect DNA delivery, differences in the cell surface architecture and capsule distribution among different *C. neoformans* serotypes (Maziarz and Perfect, 2016) may explain why the transformation efficiencies vary between strains. Furthermore, the polysaccharide capsule and melanin both confer a highly net negative charge to the cells (Nosanchuk and Casadevall., 1997), which would strongly repel DNA molecules that are usually also negatively charged.

To reduce the strength of these extramembraneous barriers, partial removal of the polysaccharide capsule was attempted by washing cells with low concentrations of DMSO before transformations. In this same vein, *C. neoformans* spheroplasts (generated by the complete removal of the polysaccharide capsule and cell wall using a cell wall degrading enzyme) was used for transformations. Despite these efforts, no positive transformants were obtained. The cells remained

viable when grown on non-selective media after DMSO wash steps and spheroplasting, suggesting that the cells were alive but not effectively transformed.

Biolistic delivery of DNA was initially designed to pass genetic material through the tough cell walls of plant cells (Klein et al., 1987). Toffaletti and co-workers (1993) demonstrated that there was a significant improvement in the efficiency of delivering DNA into serotype A strains of *C. neoformans* using biolistics as opposed to electroporation, due to higher transformation and homologous recombination efficiencies. Since then, biolistics has become the preferred transformation method used for *Cryptococcus* spp. (du Plooy et al., 2018). However, no biolistics systems were available for the transformation of *C. neoformans* for the duration of this study.

5.5 Potential drawbacks and advantages in designing the current quorum sensing biosensor in *C. neoformans*

The major drawback to using *C. neoformans* as the host strain for the whole cell-based QS biosensor is that other potential variables may influence a detected fluorescent response. Despite the CK0289 QS mutant being unable to produce mQsp1, which would bind to the selected QS promoters and induce the expression of *EGFP*, the cell is still capable of producing the Gat201, Gat204 and Liv3 TFs. As mentioned previously, all three TFs can bind to the QS-responsive promoters and may consequently affect the expression of *EGFP*. Therefore, the fluorescent signals quantified from the *C. neoformans*-based biosensor would be as a result of both TF and mQsp1 interaction. As little is known about the mechanisms involved in the cryptococcal QS response, other factors still unknown could also influence the intensity of the fluorescent signal. However, the addition of mQsp1 to the CK0289 QS mutant should still result in a more significant fluorescent response as opposed to when it is not exposed to the peptide.

Once the biosensor is constructed, and an increase in fluorescence intensity can be linked to exposure to mQsp1, the biosensor could then serve as a powerful tool to screen for compounds that may inhibit this mode of QS in the pathogen (see candidate compounds in Chapter 4). The biosensor could also be used to understand how *Cryptococcus* modulates its QS response under various conditions, such as during host infection and nutrient limitation. Because the biosensor is engineered within the pathogen that would originally synthesise and process the QS inducer, one can gauge a more accurate physiological response of the pathogen to quorum quenching drugs. Moreover, other factors potentially working in conjunction with mQsp1 would be present and influence transcription of the reporter gene more realistically. In contrast, the heterologous *Saccharomyces* system would only report on the effect QS inhibitors may have on mQsp1 and its interaction with specific QS-responsive promoters. No potential cellular countermeasures exerted by *Cryptococcus* to overcome quorum quenching drugs would be involved. Nevertheless, application of the biosensor in both yeast hosts could help improve the understanding of how *C. neoformans* survives in its environment and guide the development of new anticryptococcal drugs and treatment strategies.

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CHAPTER 6

General conclusions and recommendations

Chapter 6: General conclusions and recommendations

6.1 General discussion and conclusions

Cryptococcus neoformans is responsible for most reported cases of fungal meningitis, particularly in regions of sub-Saharan Africa where a large population of immune-compromised individuals reside (Azevedo et al., 2016; Rajasingham et al., 2017). The yeast can be found globally in both environmental and urban settings and is generally associated with the woody substrate of trees, pigeon excreta and soil (Emmons, 1951; Emmons, 1955; Gibson and Johnston, 2015). Studies investigating the effect of ecologically relevant concentrations of specific essential nutrients on the physiology of *C. neoformans* are scarce. Moreover, a small secreted peptide (Qsp1) (Lee et al., 2007) was only recently shown to play a direct role in *C. neoformans* quorum sensing (QS) and virulence (Homer et al., 2016). Thus, a major aim of this study was to determine whether nitrogen limitation (NL) impacted virulence factor expression in *C. neoformans* and if this was also affected by QS (Chapter 3). Nitrogen concentrations experienced by *C. neoformans* in its natural environment were used to simulate NL. Investigating this would provide a more accurate representation of how the yeast likely behaves in its natural environment and how this may have allowed it to adapt and acquire its pathogenic characteristics.

It was found that a decrease in nitrogen concentration resulted in an increase in polysaccharide capsule thickness, melanisation, urease activity and ergosterol biosynthesis in *C. neoformans*. Additionally, capsule thickness, melanisation and ergosterol biosynthesis were more pronounced when NL was coupled to elevated temperature. These findings confirmed previous reports that NL influences cryptococcal virulence (Lee et al., 2011; Bosch et al., unpublished). Since QS generally regulates virulence factor expression in most pathogens (Bandara et al., 2012), it was thought that QS might also be affected during NL. In accordance, the virulence phenotype of the QS mutant, *C. neoformans* CK0289, was weaker than that of the *C. neoformans* H99 wild type strain. This confirmed that QS regulated the phenotypes observed during NL. To date, no studies have investigated the mechanisms involved in regulating QS during nutrient stress in *C. neoformans.* This study has shown that nutrient stress is an important factor that may alter the QS response in *C. neoformans* and, in turn, affect virulence factor expression. Therefore, this study provides relevant preliminary data that support a link between NL, QS and virulence in *C. neoformans*.

During the test conditions used in this study, *C. neoformans* must experience alleviation from nitrogen catabolite repression (NCR) in order to metabolise the available, non-preferred nitrogen source for survival (Marzluf, 1997). Thus, key components regulating NCR may interact with components of the cell's QS system to control virulence factor expression. Pathogenicity is only observed during growth in mammalian hosts where *C. neoformans* also encounters nutrient stress, particularly in the form of NL (Ries et al., 2018). Therefore, further characterising the link between

NL, QS and cryptococcal virulence can provide insight into the yeast's pathogenicity and inform better treatment solutions. The QS peptide (Qsp1) was only able to recover the capsule phenotype of *C. neoformans* CK0289 to that of *C. neoformans* H99, suggesting that QS in *C. neoformans* may also be regulated by other metabolites. Previous studies have demonstrated that *C. neoformans* shows QS-like behaviour in response to other small, secreted peptides and pantothenic acid (Lee et al., 2007; Albequerque et al., 2014). These metabolites, and possibly other unknown ones, may thus be necessary to induce a strong QS response in *C. neoformans* combinatorially. This suggests that the current model of QS in *C. neoformans* (Homer et al., 2016), as well as the regulation of virulence, is much more complex and requires further investigation to unravel these processes.

In sub-Saharan Africa, effective cryptococcal treatment options are not always accessible (Perfect and Bicanic, 2015). Moreover, *C. neoformans* can acquire resistance to the drugs used during treatment (Smith et al., 2015; Gago et al., 2016). Therefore, alternative treatment strategies and drugs are essential. A promising alternative strategy involves using an antipathogenic approach. This strategy makes use of drugs that target non-essential cellular components, usually those involved in QS and virulence (Fong et al., 2017). A major advantage to this strategy is that it hypothetically reduces the chances of pathogens acquiring drug resistance due to a reduced selective pressure (Csermely et al., 2005; Cegelski et al., 2008; LaSarre and Federle, 2013; Kirienko et al., 2019). Moreover, previous studies have demonstrated the potential of medicinal plants as alternative sources of antipathogenic drugs against *C. neoformans* (Cardoso et al., 2017; Kumari et al., 2017; Samie et al., 2019).

In the search for novel antipathogenic drugs, it was shown that garlic-derived organosulfides inhibit *C. neoformans* growth, attenuate virulence and have multiple cellular targets (Chapter 4), highlighting the potential use of garlic for alternative anticryptococcal drug discovery and design. Specifically, the natural garlic compounds, ajoene and allicin, reduced cryptococcal capsule thickness and completely inhibited melanisation. Despite urease activity not being negatively affected after organosulfide exposure, these results require further investigation. The urease assay relies on changes in pH as a consequence of the release of ammonia during urea degradation (Cox et al., 2000; Olszewski et al., 2004). The test medium, however, is sensitive to pH changes brought about by any metabolite released by the cell and is, therefore, not specific to urease activity. An optimised assay specifically for measuring urease activity is thus required before conclusions can be drawn. Nevertheless, the garlic organosulfides still proved capable of reducing the expression of a subset of cryptococcal virulence factors. Although a group of synthetic derivatives of ajoene and allicin showed antifungal activity, their antipathogenic activity was not investigated in this study. Since they share a similar chemical backbone to the natural garlic compounds, they likely also share similar activity. Further investigation would be required to confirm this and may aid in future anticryptococcal drug design.

The fact that multiple cellular targets appear to be affected by the individual garlic compounds may prove advantageous in drug development. If a single compound is affecting multiple targets, a pathogen is less likely to acquire resistance (Espinoza-Fonseca, 2006), especially if non-essential components, such as virulence factors, are being targeted. Although rigorous clinical trials would be required to approve the safety of drugs derived from medicinal plants, this study has shown that garlic contains a group of multi-target drugs with the characteristics of *C. neoformans* growth inhibition and virulence attenuation. Therefore, further studies investigating garlic-derived organosulfides would especially be beneficial in antifungal therapy.

In order to confirm whether the observed changes in the cryptococcal virulence phenotype during NL (Chapter 3) and organosulfide treatment (Chapter 4) was linked to QS and the interference of this process, a transcription-based Qsp1-responsive yeast biosensor was designed (Chapter 5). The biosensor was also designed for the purpose of screening potential drug candidates for quorum quenching (QQ) activity against *C. neoformans*. However, after biosensor construction, no signals could be detected. Thus, only phenotypic differences between a wild type and a QS mutant of *C. neoformans* could be relied on to hypothesise on the QQ activity of garlic organosulfides and the involvement of Qsp1 in regulating virulence factor expression during NL. *Saccharomyces cerevisiae*, an ascomycetous yeast, was used as the host for the cryptococcal biosensor. Since the biosensor relied on the import of extracellularly added synthetic mature Qsp1 (mQsp1), this likely explains why no signals could be detected – *S. cerevisiae* may not have the necessary cellular machinery to detect and import Qsp1. Moreover, *S. cerevisiae* may be unable to initiate transcription from *C. neoformans* promoters. Constructing the biosensor in *C. neoformans* as the host also proved unsuccessful as no transformants could be obtained. Because little information is available on the components modulating QS in *C. neoformans*, more focused studies on this process may improve advancements in monitoring cryptococcal QS. Moreover, monitoring cryptococcal QS would greatly improve the discovery and development of novel antipathogenic drugs for alternative therapy.

6.2 Future research

This study measured phenotypic differences between a *C. neoformans* wild type and QS mutant strain to determine if ecologically relevant nitrogen concentrations affected QS and virulence. In order to more comprehensively characterise this effect, bioinformatics tools such as RNA sequencing can be employed. This will allow for the characterisation of specific sets of genes that are expressed during treatments (Wang et al., 2009). These profiles could help develop further understanding of the physiological behaviour of *C. neoformans* likely displayed in its natural environment. Furthermore, RNA sequencing could aid in establishing whether QS via Qsp1 does regulate virulence factor expression as well as other responses during NL. Also, the influence of combinations of Qsp1 with other potential QS metabolites on pathogenicity during NL could be

investigated. To relate pathogenic responses during NL to a medical setting, *in vivo* studies should be performed using nitrogen concentrations that are present during host infection.

Our study demonstrated that QS is necessary to induce ergosterol biosynthesis in *C. neoformans* during NL. Koselny et al. (2018) indicated that there was a positive correlation between pyroptosis-induced lysis of macrophages and the ergosterol content in the cell membranes of *S. cerevisiae*, *C. albicans* and *C. neoformans*. Pyroptosis is a form of programmed cell death in response to severe inflammation (Bergsbaken et al., 2009). Although ergosterol is primarily located within the cell membrane and not the cell surface, hence inaccessible to macrophages, it was shown that this sterol could be exported via cell wall mannoproteins (Koselny et al., 2018). Additionally, the major component of extracellular vesicles secreted by yeasts like *Cryptococcus* consists of ergosterol (Rodrigues et al., 2007). Thus, cell surface receptors of macrophages can encounter ergosterol, bind to these molecules and undergo pyroptosis. Therefore, if Qsp1 is required for increased ergosterol biosynthesis during elevated temperatures, such as within the human host, inhibition of QS could lower cell membrane ergosterol and reduce the exposure of macrophages to this sterol. This could potentially prolong the activity of macrophages against *Cryptococcus* and promote pathogen clearance. More research can be done to investigate this and may provide an effective alternative to directly targeting ergosterol biosynthesis in *C. neoformans* as anticryptococcal therapy.

Biolistics can be used to transform the QS biosensor constructs into *C. neoformans* efficiently. The *C. neoformans* strains used in this study are serotype A strains, which show greater success in genetic transformation using biolistics than electroporation (Toffaletti et al., 1996; Lin et al., 2015). A *C. neoformans* QS biosensor would serve as an important diagnostic tool in antipathogenic drug discovery and design. Moreover, the biosensor could be used to assess whether the other cryptococcal QS molecules, such as Qsp2 and pantothenic acid, also affect similar targets of Qsp1. The approach used to construct the *C. neoformans* QS biosensor in this study could also be used in a similar manner to design a QS biosensor for other pathogenic fungi such as *Candida albicans*.

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APPENDIX

Table A5.1: Strains constructed in this study

Strain number	Strain	Vector	Recombinant strain name
1	*E. coli* DH5α	YIp-*PGK1-EGFP*	DH5α_ YIp-*PGK1-EGFP*
2	*E. coli* DH5α	YIp-*GAT201-EGFP*	DH5α_ YIp-*GAT201-EGFP*
3	*E. coli* DH5α	YIp-*GAT204-EGFP*	DH5α_ YIp-*GAT204-EGFP*
4	*E. coli* DH5α	YIp-*QSP1-EGFP*	DH5α_ YIp-*QSP1-EGFP*
5	*E. coli* DH5α	YIp-*GAL7-EGFP*	DH5α_ YIp-*GAL7-EGFP*
6	*E. coli* DH5α	pSD-*GAT201-EGFP*	DH5α_ pSD-*GAT201-EGFP*
7	*E. coli* DH5α	pSD-*GAT204-EGFP*	DH5α_ pSD-*GAT204-EGFP*
8	*E. coli* DH5α	pSD-*QSP1-EGFP*	DH5α_ pSD-*QSP1-EGFP*
9	*E. coli* DH5α	pSD-*GAL7-EGFP*	DH5α_ pSD-*GAL7-EGFP*
10	*S. cerevisiae* Y294	YIp-*PGK1-EGFP*	Y294_YIp-*PGK1-EGFP*
11	*S. cerevisiae* Y294	YIp-*GAT201-EGFP*	Y294_YIp-*GAT201-EGFP*
12	*S. cerevisiae* Y294	YIp-*GAT204-EGFP*	Y294_YIp-*GAT204-EGFP*
13	*S. cerevisiae* Y294	YIp-*QSP1-EGFP*	Y294_YIp-*QSP1-EGFP*
14	*S. cerevisiae* Y294	YIp-*GAL7-EGFP*	Y294_YIp-*GAL7-EGFP*

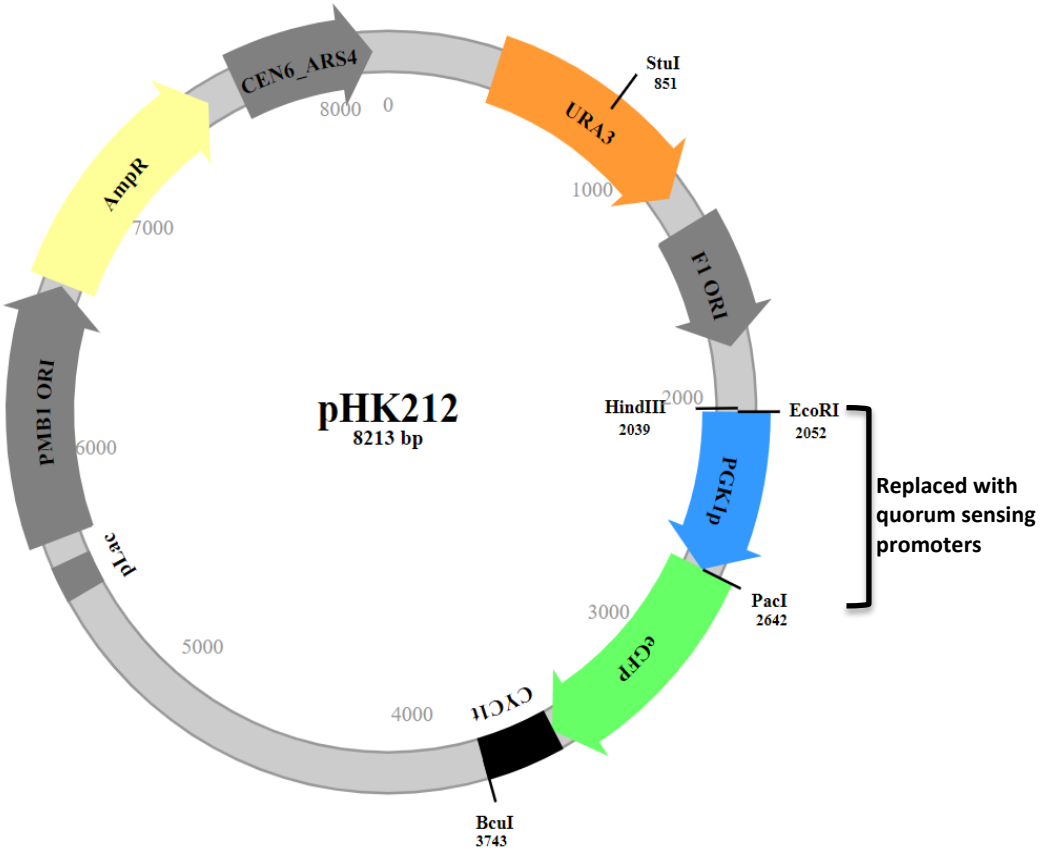

Figure A5.1: Plasmid map of the intermediary pHK212 centromeric vector containing the *EGFP* reporter gene flanked by the *Saccharomyces cerevisiae PGK1* promoter and CYC1 terminator. Positions of relevant restriction enzyme sites are annotated with black lines. The vector map was generated using Angular Plasmid (https://github.com/vixis/angularplasmid/).

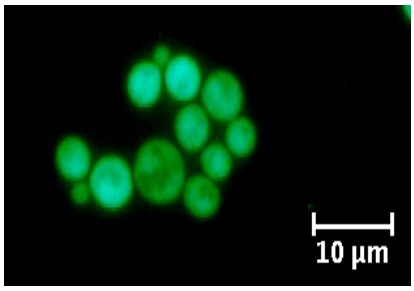

Figure A5.2: Fluorescence micrograph of the *Saccharomyces cerevisiae* Y294 positive control strain containing the YIp-*PGK1-EGFP* vector. Scale bar: 10 µm.