The development of a CRISPR-Cas9 gene editing system for *Cryptococcus deneoformans*

By

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DISSERTATION SUMMARY

The pathogenic yeasts, *Cryptococcus neoformans* and *C. deneoformans*, are responsible for potentially fatal meningoencephalitis in immunocompromised individuals, most notably in patients who have AIDS. Only three drugs are commonly administered to treat cryptococcal infections and most are not readily available in developing countries most affected by the AIDS pandemic. Cheaper and more widely available drugs are therefore needed. Developing molecular methods to disable genes encoding virulence factors could help to elucidate the mechanism of action of potential drugs against these fungal pathogens. Previously, researchers mostly relied on biolistic transformation to deliver DNA into cells for homologous integration into the targeted site. Recent developments with CRISPR-Cas9-based systems for gene targeting made it possible to utilise another transformation technique, electroporation, to knock genes out. In this study, a one-step CRISPR-Cas9 system was developed to be delivered into cells with electroporation. This system consists out of two plasmids, carrying a nourseothricin and G418 resistance marker respectively, as well as a *CAS9* gene. A third plasmid was used to construct guide DNA, which was then amplified and cloned into the two CRISPR-Cas9 plasmids carrying the *CAS9* gene. The plasmids carrying the CRISPR-Cas9 components were maintained transiently for expression of the CRISPR-Cas9 genes before these constructs were degraded by the cells. Donor DNA was also constructed to remove parts of the biosynthetic genes *ADE2* and *HIS3* to obtain adenine and histidine auxotrophic mutants with visually discernible phenotypes. A second round of transformation can then introduce new donor DNA to repair these auxotrophic genes whilst disrupting virulence genes for virulence studies. Electroporation proved to be very inefficient in this study and gene targeting was unsuccessful. Using large amounts of plasmid and donor DNA yielded the best results, although no more than 8 colonies were seen on a few selective media agar plates. Inefficient transformation could be due to old and faulty electroporation equipment or ineffective delivery of the electrical current to cells. In the future, other transformation methods will be employed to deliver the plasmids constructed in this study into *C. deneoformans* cells. This system can then be used to remove virulence genes to study their role in infection, which could help to elucidate the mechanism of

action behind potential drugs. For instance, a capsule-less mutant could reveal what effect a drug targeting capsule synthesis will have on the ability of these yeasts to cause disease.

Keywords: AIDS; Biolistic transformation; CRISPR-Cas9; *Cryptococcus*; Donor DNA; Electroporation; Gene targeting; Homologous recombination; Pathogenic yeast

DECLARATION

I, Lukas Marthinus du Plooy, declare that the Master's Degree research dissertation or publishable, interrelated articles, or coursework Master's Degree mini-dissertation that I herewith submit for the Master's Degree qualification at the University of the Free State is my independent work, and that I have not previously submitted it for a qualification at another institution of higher education.

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LIST OF ABBREVIATIONS

A – Adenine

AIDS – Acquired Immune Deficiency Syndrome

bp – Base Pair

CAS – CRISPR Associated

CD4 – Cluster of Differentiation 4

CNS – Central Nervous System

CRISPR – Clustered Regularly Interspaced Short Palindromic Repeats

C – Cytosine

DSB – Double-Strand Break

DSBR – Double-Strand Break Repair

DNA – Deoxyribonucleic Acid

dsDNA – Double-Stranded DNA

G418 – Geneticin

gDNA – Guide DNA

gRNA – Guide RNA

G – Guanine

GXM – Glucuronoxylomannan

GXMGal – glucuronoxylomannogalactan

HDV – Hepatitis Delta Virus

HIV – Human Immunodeficiency Virus

HJ – Holliday Junction

HR – Homologous Recombination

HsCAS9 – Human codon-optimised *CAS9*

LB – Lysogeny Broth

NEB® – New England Biolabs

NHEJ – Non-homologous End Joining

OD – Optical Density

ORF – Open Reading Frame

PCR – Polymerase Chain Reaction

PEG – Polyethylene Glycol

PIKK – Phosphoinositide-3-kinase-related Protein Kinase

PVDF – Polyvinylidene Difluoride

RNA – Ribonucleic Acid

SDS-PAGE – Sodium Dodecyl Sulphate – Polyacrylamide Gel Electrophoresis

ssDNA – Single-Stranded DNA

TALEN – Transcription Activator-like Effector Nuclease

T – Thymine

UNAIDS – Joined United Nations Programme on HIV/AIDS

U – Uracil

UV – Ultra-violet

WHO – World Health Organisation

YNB – Yeast Nitrogen Base

YPD – Yeast Extract, Peptone and D-glucose medium

YPGalactose – Yeast Extract, Peptone and Galactose medium

ZFN – Zinc Finger Nuclease

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promoter, terminator and the *HsCAS9* gene. Lanes 5 to 6 show the same for the pSDMA58 plasmid. All of these plasmids were digested with *Bam*HI.

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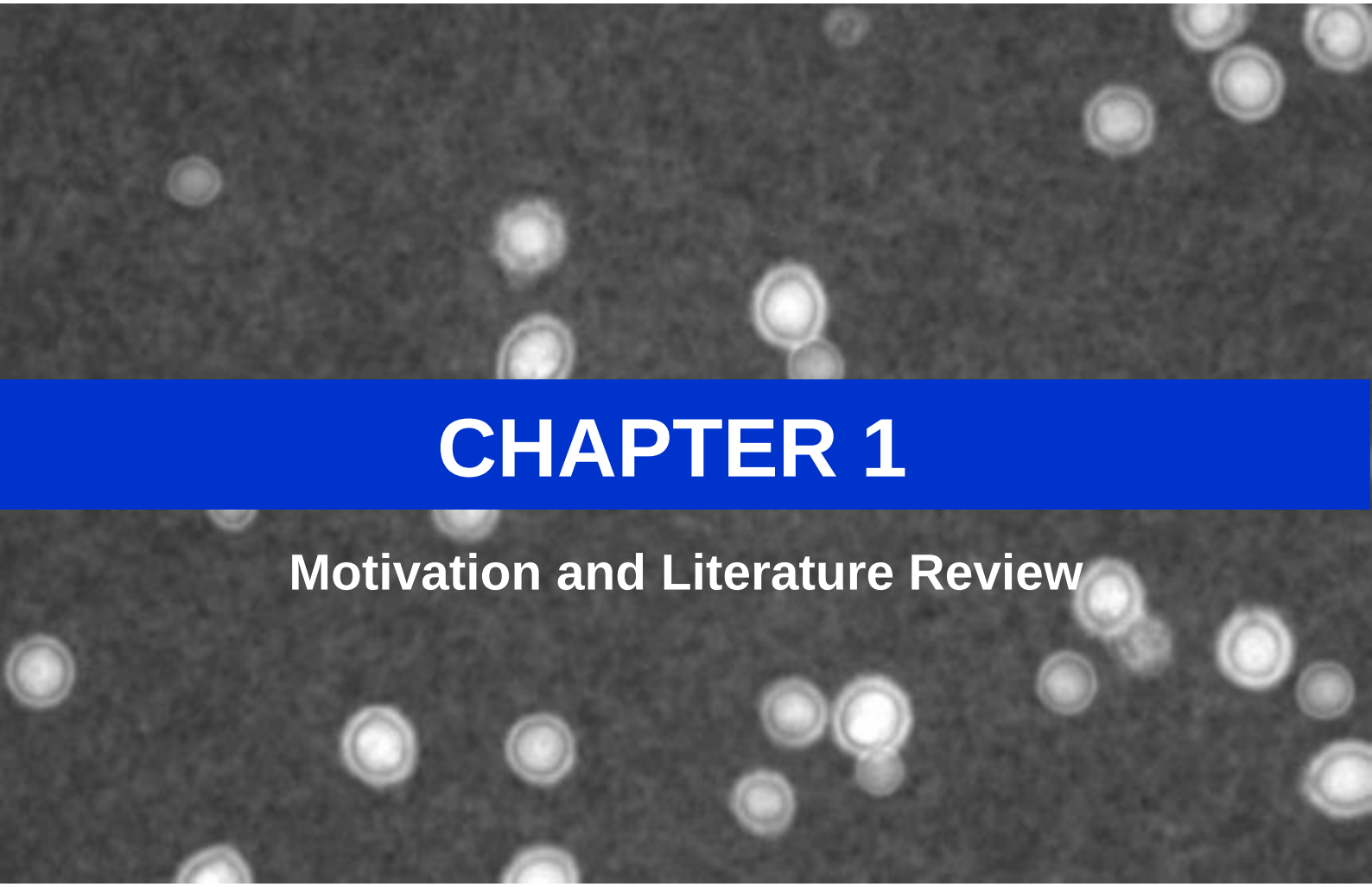

CHAPTER 1

Motivation and Literature Review

SECTION A

1. Motivation

The immunosuppressing disease, AIDS (acquired immune deficiency syndrome), is regarded as the worst global disaster to hit the modern world (Carroll & Boseley, 2004; Carter, 2008). The human immunodeficiency virus (HIV), the causative agent of AIDS, infects and destroys $CD4^+$ (cluster of differentiation 4) T-helper cells, which severely impairs immune function (Geijtenbeek *et al*., 2000). This viral disease was responsible for 940 000 deaths and 1.8 million new infections worldwide in 2017 alone, which brings the total of HIV-positive individuals to 36.9 million as reported by the Joint United Nations Programme on HIV/AIDS (UNAIDS). Around 110 000 of these deaths were from South Africa (UNAIDS report), which forms part of Sub-Saharan Africa, the most affected area (Symington *et al*., 2017). AIDS opens the door to infections rarely seen in immunocompetent individuals and for which treatments are consequently not widely available.

Cryptococcosis, caused by yeasts of the *Cryptococcus neoformans*/*gattii* species complex, is the leading mycological contributor to AIDS-related deaths. These yeasts enter the host via the lungs and spread to the central nervous system (CNS), which leads to meningitis or potentially fatal meningoencephalitis if left untreated (Idnurm *et al.*, 2005; Sabiiti *et al*., 2014). Being regarded as opportunistic pathogens (and true pathogens, in the case of the *C. gattii* species complex) these yeasts occur readily in the environment, such as in bird excreta and on the bark of trees (May *et al.*, 2016). Immunocompromised individuals are therefore frequently and unavoidably exposed to spores and desiccated cells, which are thought to be the infectious propagules (DeLeon Rodriguez & Casadevall, 2016).

Cryptococcal infection is usually the first indication of AIDS (Mitchell & Perfect, 1995) and cryptococcosis in humans only became a major health threat with the onset of AIDS (May *et al*., 2016). A 1500% increase in cryptococcosis was observed in the period from 1981 to 1990 in the United States due to the AIDS epidemic

(Antinori, 2013). This disease is however not only of concern to HIV-positive individuals, but frequently affects other immunocompromised patients as well. Various studies have shown that old age, cancer, solid organ transplants, diabetes mellitus, corticosteroid therapy and other conditions that compromise T-cell mediated immunity are all risk factors for cryptococcosis (Kauffman, 2001; Kishi *et al*., 2006). Cryptococcosis is the third most common invasive fungal infection among solid organ transplant recipients after candidiasis and aspergillosis (Vilchez *et al*., 2002; Pappas, 2013) and 20% of cryptococcosis patients with no other risk factor are elderly (Kauffman, 2001).

Immunocompetent persons are also not spared from infection, especially those caused by *C. gattii* (Idnurm *et al.*, 2005; Gago *et al.*, 2017). In 1999, an outbreak of *C. gattii* occurred in British Columbia, affecting several hundred otherwise healthy individuals (Voelz *et al.*, 2014; Acheson *et al.*, 2017). Several cases of *C. neoformans* (previously known as *C. neoformans* variety *grubii*, see Hagen *et al*., 2015) infections have also been reported in immunocompetent individuals without any apparent risk factors, although these infections seem to occur much less often than *C. gattii* infections (Chen *et al.*, 2008; Pappas, 2013). In contrast, when including immunocompromised patients in the picture, the majority of all cases are caused by *C. neoformans*, with infections caused by species of the *C. gattii* complex and *C. deneoformans* (previously known as *C. neoformans* variety *neoformans*) as well as other species in the genus being much less prevalent (Ikeda *et al*., 2002; Gago *et al.*, 2017).

The *C. neoformans*/*gattii* species complex has an extensive arsenal of virulence factors which all contribute to the successful pathogenicity of these yeasts (Coelho *et al.*, 2014). Studying these virulence factors could help to identify new drug and vaccine targets and could elucidate what contribution each virulence factor makes to the pathogenicity of an organism. This could in turn help in the search for virulence factors in other opportunistic and emerging pathogens through gene homology.

Cheaper and more effective drugs are needed to improve the life expectancy of AIDS patients in developing countries. Cryptococcosis is currently treated with three off-patent and dated drugs, amphotericin B (and its liposomal derivatives), 5-fluorocytosine and fluconazole, which frequently still do not reach the most affected developing countries (Perfect, 2013; Perfect & Bicanic, 2015). Fluconazole, a fungistatic drug, is however donated and distributed through the Pfizer Inc. Diflucan Partnership Program in many affected developing countries (Wertheimer *et al*., 2004). The suppressive nature of this drug necessitates continuous administration, which frequently leads to relapse in patients due to interactions with other drugs, poor compliance with treatment, malabsorption or the development of drug resistance with long term use (Armengou *et al*., 1996). Although the Diflucan Partnership Program is of immense help, the development or discovery of a cheap and more efficient drug could contribute significantly to the fight against this disease.

Studying the function of virulence genes could help to elucidate the mechanism of action of potential drugs and could, therefore, contribute to the development of new drugs. For instance, if a new drug is found to disrupt a certain cellular trait in a screening process, mutants lacking the trait can be constructed and studied to determine if, and how, this trait affects virulence. Studying gene function could also elucidate how the expression of virulence genes are regulated and could reveal genes working in unison during infection. To study the function of virulence genes a reliable gene targeting strategy should be available. The recently developed genetic engineering system, CRISPR-Cas9, could be such a strategy.

SECTION B

This section of the chapter was published in *Frontiers in Microbiology*, following the reference style of the journal. Therefore, repetition of some information elsewhere in this dissertation could not be avoided.

The candidate, Lukas Marthinus du Plooy, conducted the literature study and wrote the manuscript. The supervisor and co-supervisors provided editorial and grammatical input.

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REVIEW
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Functional Characterization of Cryptococcal Genes: Then and Now

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Site-directed mutagenesis enables researchers to switch a gene of interest off for functional characterization of the gene. In the pathogenic yeasts, *Cryptococcus neoformans* and sister species *C. deneoformans*, this is almost exclusively achieved by introducing DNA into cells through either biolistic transformation or electroporation. The targeted gene is then disrupted by homologous recombination (HR) between the gene and the transforming DNA. Both techniques have downsides; biolistic transformation equipment is very expensive, limiting the use thereof to well-resourced laboratories, and HR occurs at extremely low frequencies in electroporated cryptococcal cells, making this method unappealing for gene targeting when not making use of additional modifications or methods to enhance HR in these cells. One approach to increase the frequency of HR in electroporated cryptococcal cells have recently been described. In this approach, CRISPR-Cas9 technology is utilized to form a double strand break in the targeted gene where after the occurrence of HR seems to be higher. The less expensive electroporation technique can therefore be used to deliver the CRISPR-Cas9 components into cells to disrupt a gene of interest, but only if the CRISPR components can be maintained for long enough in cells to enable their expression. Maintenance of episomal DNA occurs readily in *C. deneoformans*, but only under certain conditions in *C. neoformans*. In addition, CRISPR-Cas9 allows for gene complementation in order to fulfill Falkow's molecular Koch's postulates and adds other novel methods for studying genes as well, such as the addition of a fluorophore to an inactive Cas9 enzyme to highlight the location of a gene in a chromosome. These developments add less expensive alternatives to current methods, which could lead to more research on this yeast in developing countries where cryptococcal infections are more prevalent and researchers have access to more clinical isolates.

Keywords: biolistic transformation, CRISPR-Cas9, *Cryptococcus,* electroporation, gene targeting

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INTRODUCTION

Site-directed mutagenesis is an essential tool for the functional characterization of genes and is therefore also used to identify virulence genes in pathogens. It involves disrupting or altering a gene of interest by exploiting homologous recombination (HR), the double strand break (DSB) repair machinery of cells, where the separated ends are joined by recombination with a homologous strand (Aylon and Kupiec, 2004). For gene disruption, a synthetic oligonucleotide, often referred to as donor DNA, is introduced into a cell which is then incorporated into the gene through HR

(Kuwayama et al., 2008; Carrigan et al., 2011). Depending on the donor DNA utilized, disruption can be obtained through a frameshift mutation where an inactive or altered peptide is produced, or a deletion can be obtained with the insertion of a long stretch of bases, such as a reporter gene, resulting in the production of no protein at all. One can thereafter determine the change in the phenotype and ultimately deduce the function of the gene product. Before HR can take place, the donor DNA needs to be delivered into cells, which is often a more laborious process for cells with a thick cell wall or capsule, such as plant and fungal cells. This is also true for the yeasts *Cryptococcus neoformans* and *C. deneoformans*, the causative agents of cryptococcosis in immunocompromised patients (Goins et al., 2006).

Cryptococcosis is an infection of the pulmonary system of humans and other mammals and if untreated, the disease could progress to cause an often deadly inflammatory condition of the brain and spine (Nielsen et al., 2007; Djordjevic, 2010). *Cryptococcus neoformans* was first isolated between 1894 and 1895 by Busse and Buschke from a lesion of a woman's tibia (Barnett, 2010; Espinel-Ingroff and Kidd, 2015). It was not yet possible to study the molecular mechanisms behind the pathogenesis of this yeast for close to century after this discovery, despite knowledge of its virulence.

All of the pathogenic *Cryptococcus* species were initially classified as varieties of a single species, *C. neoformans* (Espinel-Ingroff and Kidd, 2015). In 2002, molecular characterization and other work led to the recognition of *C. neoformans* var. *gattii* as the distinct species *C. gattii* (Kwon-Chung et al., 2002; Kwon-Chung and Varma, 2006). Another reclassification was proposed by Hagen et al. (2015) following a debate between proponents of the old classification and a revised classification of these yeasts. *Cryptococcus neoformans* var. *neoformans* (also commonly referred to as serotype D) was renamed to *C. deneoformans* and *C. neoformans* var. *grubii* (serotype A) retained the name *C. neoformans*. The five genotypes of *C. gattii* were also raised to species level, yielding a total of seven species in the new *C. neoformans*/*gattii* species complex.

In the 1980s, virulence studies on *C. neoformans* and *C. deneoformans* were done using non-specific mutagenesis, most notably by the Kwon-Chung group. Mutants lacking the most apparent virulence traits (i.e., capsule and melanin formation as well as growth at 37°C) were generated with UV irradiation and subsequent cloning (Kwon-Chung et al., 1982; Rhodes et al., 1982; Kwon-Chung and Rhodes, 1986). However, nothing about the molecular mechanisms behind these mutants were known, and site-directed mutagenesis only became possible in *Cryptococcus* species when Edman and Kwon-Chung (1990) adopted an electroporation protocol optimized for *Saccharomyces cerevisiae* to introduce foreign DNA into the cells. With this technique, cell membranes are made more permeable by exposure to an electrical impulse to facilitate the transport of particles, such as DNA, across the membranes (Neumann et al., 1982). Electroporation was first developed by Neumann et al. (1982) for the transfection of mouse lyoma cells and could deliver DNA into cryptococcal cells more readily than chemical transformation methods used for *S. cerevisiae*, such as the lithium acetate method described by Ito et al. (1983). In fact, to our knowledge the only reference in literature made to a chemical transformation method of cryptococcal cells was by Ou et al. (2011), who used a "lithium acetate yeast transduction kit" to introduce DNA into *C. neoformans*. Chemical transformation methods are very inefficient in these yeasts, due to the thick capsule and cell wall that must be crossed by the transforming DNA (Srikanta et al., 2014).

A second technique, biolistic transformation, involves ballistically transforming cells with DNA-coated metal microparticles (Klein et al., 1987; Toffaletti et al., 1993). Biolistic transformation was originally developed by Klein et al. (1987) to transfect plant cells in hopes of circumventing some of the limitations faced with delivering DNA into these cells, such as getting enough DNA through the thick cell wall. Since fungi are also covered with a thick cell wall, this technique is also frequently used to deliver DNA into fungal cells, such as *Trichoderma harzianum* and *Gliocladium virens* (Lorito et al., 1993). As was the case with electroporation, a biolistic transformation protocol was borrowed from *S. cerevisiae* and adapted for *C. neoformans* by Toffaletti et al. (1993). This method yields higher transformation and HR efficiencies than electroporation and have since been established as the method of choice by many *Cryptococcus* researchers (Lin et al., 2014; Srikanta et al., 2014). Other attempts at transforming these yeasts include protoplasting and *Agrobacterium*-mediated transformation (AMT) (McClelland et al., 2005; Lin et al., 2014; Srikanta et al., 2014). *Agrobacterium tumefaciens* is a gram-negative soil bacterium that is capable of transferring a Ti plasmid vector carrying the T-DNA (transfer DNA) into plant and fungal cells for integration into a host chromosome (McClelland et al., 2005; Srikanta et al., 2014). Both techniques are very ineffective in achieving site-directed mutagenesis and is therefore not used for gene characterization (Lin et al., 2014). *Agrobacterium*-mediated transformation is less time consuming than preparing protoplasts (McClelland et al., 2005) and does yield high transformation efficiency and stable transformants, but does not mediate HR (Srikanta et al., 2014).

RNA-MEDIATED GENE SILENCING

An alternative to the DNA targeting techniques described above is to target the transcription product, messenger RNA (mRNA), instead to ultimately elucidate the role of the relevant gene. Napoli et al. (1990) discovered that supplementing petunia plants (*Petunia* spp.) with an additional copy of the chalcone synthase (CHS) gene, one of the genes responsible for the violet pigment in petunia flowers, unexpectedly yielded white flowers instead. They concluded that the transferred gene somehow caused both the endogenous and transferred gene to be suppressed. Further studies revealed that introducing a double-stranded RNA (dsRNA) sequence homologous to a sequence in a cell results in silencing of the gene (Fire et al., 1998). It was initially thought that silencing occurred when the antisense strand bound to complementary mRNA, marking it for degradation. Two independent groups (Hammond et al., 2000; Zamore et al., 2000)

showed that this was not entirely the case; an enzyme processes dsRNA into small interfering RNA (siRNA) of about 21–23 nucleotides. The enzyme, known as Dicer, was later identified by Bernstein et al. (2001). Dicer, a member of the RNase III family, therefore digests dsRNA into mature siRNAs. Further work showed that these short siRNA molecules then enter an assembly pathway with effector assemblies known as RNA-induced silencing complexes (RISCs), which facilitates duplex unwinding by a protein known as argonaute protein (Carthew and Sontheimer, 2009). This RNA-protein complex is then responsible for the sequence specific cleavage of mRNA using the siRNA as guide (Skowyra and Doering, 2012).

This cellular process can therefore be exploited to silence the expression of targeted genes by introducing a dsRNA molecule homologous to the mRNA of a targeted gene into cells. This dsRNA molecule can be synthesized *in vivo* or *in vitro* and then introduced into cells with electroporation (Liu et al., 2002). Gorlach and co-workers discovered in 2002 that RNA-mediated gene silencing functions in both *C. neoformans* and *C. deneoformans* (Skowyra and Doering, 2012). They successfully suppressed expression of the calcineurin A (*CNA1*) gene in *C. deneoformans* and laccase (*LAC1*) gene in *C. neoformans*. Another group, Liu et al. (2002), suppressed *CAP59*, a gene involved in capsule synthesis and *ADE2,* a gene in the adenine biosynthetic pathway in *C. deneoformans*. RNA interference (RNAi) have several advantages over conventional gene disruption techniques relying on HR (Skowyra and Doering, 2012). For instance, the *in vivo* synthesis of the dsRNA can be driven by various promoters, such as inducible promoters which adds more control over when a gene is targeted. However, the effect of a gene is not entirely eliminated with RNAi as is the case with gene deletion via HR; genes are "knocked down" instead of "knocked out" (Skowyra and Doering, 2012). The level of expression after RNA-mediated gene silencing depends on a number of factors, including the kinetics and stability of gene expression and the efficiency of the interference. This property can, however, be exploited to determine the function of essential genes, which would not be possible with gene deletion techniques.

ELECTROPORATION VS. BIOLISTIC TRANSFORMATION

Both electroporation and biolistic transformation are frequently used molecular techniques when studying *C. neoformans* and *C. deneoformans* (Lin et al., 2014; Wang et al., 2016). However, gene targeting only became widespread when biolistic transformation was established (Lin et al., 2014; Srikanta et al., 2014). This technique has since been established as the preferred method for transforming these yeasts and significant progress have been made in identifying the genes behind some of the more prominent cryptococcal virulence factors using this method, such as the identification of genes that play a role in growth at 37°C. In fact, the first virulence gene replaced through biolistic transformation was the *n*-myristyl transferase (*NMT1*) gene which resulted in an avirulent temperature sensitive myristic acid auxotroph (Lodge et al., 1994; Perfect, 2006). Between 17.5 and 100% of transformants obtained with biolistic transformation are stable, while the majority of transformants obtained with electroporation are unstable; especially when auxotrophic markers are used (Lin et al., 2014). Electroporated cells transformed with these markers tend to lose the plasmid vectors after only a few generations, even when maintained on selective media (Varma and Kwon-Chung, 1992). This indicates that these vectors are maintained episomally instead of being integrated into the genome, either ectopically or through HR. Drug resistance markers generally yield better results with electroporation and Varma and Kwon-Chung (2000) achieved a 100% stability by using a cycloheximide resistance marker for selection. The transformation efficiency was low, however, and it was proposed that genomic integration was a requirement in this case for the cells to survive selection.

Homologous recombination did not necessarily occur in all stable transformants, which is required to obtain mutants lacking the targeted gene product. Biolistic transformation yields a HR frequency of between 2 and 50% in *C. neoformans* (Davidson et al., 2000). It has been shown that HR varies depending on the gene and strain, making frequencies between 1 and 10% more typical (Lin et al., 2014). In congenic *C. deneoformans* strains biolistic transformation yields a HR frequency of ∼1–4% (Davidson et al., 2000). In contrast, the HR frequency obtained with electroporation varies between 0.00001 and 0.001% for *C. deneoformans* (Davidson et al., 2000). Electroporation alone in *C. neoformans* is very inefficient and is therefore generally not applied to this yeast without help from other techniques to increase the frequency of HR. Toffaletti et al. (1993) obtained no stable transformants using only electroporation. However, Lin et al. (2014) had some success, where HR occurred in 2 out of 140 stable transformants when a G418 (geneticin) resistance marker was used; whereas no HR occurred in a total of 15 stable transformants when a nourseothricin resistance marker was used in genetically identical cells. It is therefore clear that the type of selection marker plays a role in the success of electroporation. The less favorable outcome of electroporation has been attributed to the inability of this technique to deliver DNA to the nucleus (Davidson et al., 2000). This does not, however, explain how electroporated stable transformants can grow on selective media without being able to migrate the DNA to the nucleus in order to express the selection marker.

Various modifications were made to plasmid vectors or cryptococcal cells to enhance stability or the frequency of HR, especially when using electroporation as a transformation method. The presence of an autonomously replicating sequence (ARS), obtained by the interaction of transforming DNA with the host genome, has enhanced the maintenance of plasmid vectors as extrachromosomal plasmids (Varma and Kwon-Chung, 1992, 1998). Varma and Kwon-Chung (1998) isolated such an ARS-like sequence, referred to as a "STAB" element, from a minichromosome obtained from stable electroporated cryptococcal cells and added it to otherwise unstable plasmids to enhance stability. It was later shown that this sequence originated from *Escherichia coli* and had no effect on the stability of transformants (Hull and Heitman, 2002). Telomeric repeats

added to the end of a linearized vector did, however, increase stability of electroporated transformants (Edman, 1992).

More recent improvements include Ku mutants and the use of split-markers for selection which both lead to a higher HR frequency. The Ku mutant approach by Goins et al. (2006) involves deleting the genes encoding the Ku70–Ku80 heterodimer, which play a role in non-homologous end-joining (NHEJ), another cellular process responsible for repairing DSBs. This DNA repair process seems to be the preferred process in *C. neoformans* and *C. deneoformans*, explaining the low HR frequencies seen in these pathogens, even when biolistic transformation is employed (Arras and Fraser, 2016). The inability to repair DSBs with NHEJ increases the frequency of HR to almost 100%, although Ku mutants show altered virulence in mice and expression of the *KU80* gene is upregulated during human infection, making Ku mutants unsuitable for virulence studies (Arras et al., 2016). However, the use of chemical inhibitors of the NHEJ pathway could circumvent these effects on virulence. Arras and Fraser (2016) tested eight inhibitors of mammalian NHEJ and found that four influenced the rate of HR for multiple targeted genes in *Cryptococcus neoformans*. N-(6-aminohexyl)-5-chloro-1-naphthalenesulfonamide (W7), an inhibitor of the production of the Ku cofactor inositol hexakisphosphate, performed the best and is relatively inexpensive. In the split-marker approach, the selection marker is split into two fragments, requiring recombination to function (Fu et al., 2006). The likelihood of two additional recombination events occurring in the targeted gene is thereafter higher and increases the frequency of HR up to eight times when *URA5* is used as a selection marker.

Biolistic transformation is clearly the better technique, especially in *C. neoformans*, while electroporation can still be used to transform wild type C. *deneoformans* if HR is not a requirement. Inhibitors of NHEJ can be used to achieve HR with electroporation of cryptococcal cells, although this approach have thus far not been widely adopted. *Cryptococcus neoformans* is responsible for more than 90% of all cryptococcal infections worldwide and has the highest growth rate at 37°C of all *Cryptococcus* species, making this species the most virulent (Litvintseva et al., 2011; Hagen et al., 2015). It therefore makes sense to do virulence studies on *C. neoformans* species instead of *C. deneoformans*. Biolistic transformation is, however, generally more expensive than electroporation. The Biolistic® PDS-1000/He Particle Delivery System sold by Bio-Rad Laboratories, Inc. was the first commercially available system and has been established as the most frequently used system by 1998 (Kikkert, 1993; Hagio, 1998). Most of the molecular work on *Cryptococcus* species. involving biolistic transformation most frequently rely on the Bio-Rad system since the first protocol for biolistic transformation of cryptococcal cells made use of helium for particle delivery, the approach this system was based on (Toffaletti et al., 1993). The Bio-Rad Biolistic® PDS-1000/He Particle Delivery System costs US$ 33,000 compared to US$ 8,245 (listed prices as on June 2018) for a Gene Pulser Xcell™ Electroporation System also sold by Bio-Rad Laboratories, Inc. This high price is furthermore accompanied by expensive consumables, such as gold beads, macrocarriers, stopping screens, and rupture disks (Lin et al., 2014), whereas the only additional equipment required for electroporation is reusable electroporation cuvettes. This restricts the use of the biolistic method to well-resourced laboratories. Slightly cheaper apparatus could, however, be obtained, such as hand-held gene guns and bench-top particle delivery systems from other manufacturers under-represented in literature as well as components for do-it-yourself (DIY) particle delivery systems (Hanson et al., 2016), although the latter option is accompanied by trade-offs involving safety, control over bombardment power, and consistency from transformation to transformation.

THE CRISPR-CAS9 REVOLUTION

The development of a CRISPR-Cas9 system for gene targeting brought about a major breakthrough in genetic engineering, allowing researchers to target genes more accurately than ever before (McCarthy and Walsh, 2017). CRISPR, or Clustered Regularly Interspaced Short Palindromic Repeats, and the associated genes (*CAS*-genes) is a set of genes used by prokaryotes to protect themselves against invading genetic elements, such as viruses and plasmids (Van der Oost et al., 2009). This is found in over 88% of all archaeal genomes and 30% of bacterial genomes. Ishino et al. (1987) first noticed the CRISPR array in *E. coli* but paid little further attention to these repeats. The function only became a subject of research when Mojica et al. (1993) discovered similar repeats in *Haloferax mediterranei* and finally reported on their origin and possible function in 2005 (Mojica et al., 2005).

Further research revealed that foreign DNA are digested upon entering prokaryotic cells and integrated as "spacers" between two palindromic repeats in the CRISPR array by the Cas1 and Cas2 proteins (in most cases) in the acquisition phase (Levy et al., 2015; Makarova et al., 2015). Both the spacers and repeats are about 20–50 base pairs in length and new spacers are integrated next to a 100–500 base pair AT-rich region referred to as the leader sequence, which is believed to serve as a promoter for CRISPR transcription (Nuñez et al., 2014; Zhang and Ye, 2017). During the expression phase, the CRISPR array is transcribed and processed to mature CRISPR RNAs (crRNA) consisting of a spacer and one of the adjacent repeats (Arras et al., 2016). A single crRNA then associates with a Cas protein or protein complex and guides the effector complex to a complementary sequence on invading DNA, where the Cas nuclease creates a DSB during the interference phase (Zhang and Ye, 2017). The Cas protein or protein complex recognizes a ∼2–4 base pair protospacer adjacent motif (PAM) sequence on the invading DNA which is absent from the spacer to prevent digestion of the CRISPR array (Nuñez et al., 2014). The diverse CRISPR systems are classified into two classes: class 1, if a multi-subunit protein complex is involved in the interference phase and class 2, if a single protein is responsible for the interference phase (Zhang and Ye, 2017). These systems are further subdivided into types based on the signature proteins in the system.

The potential of this molecular immune system for gene targeting became apparent when it was discovered that the Cas nuclease was a programable restriction enzyme

(Marraffini and Sontheimer, 2008). This notion was reinforced when Jinek et al. (2012) showed that Cas9 could cut DNA *in vitro* and that the enzyme can be programmed with custom-designed crRNAs. This group also showed that crRNA and trans-activating crRNA (tracrRNA – a short RNA molecule involved in pre-crRNA processing and binding to Cas9) can be joined into a single guide RNA (sgRNA or gRNA), simplifying the system for use by researchers. This was soon followed by the first *in vivo* use of CRISPR-Cas9 in eukaryotes by Cong et al. (2013), who used this technology to target genes in human and mouse cells. CRISPR-Cas9 was quickly adopted and modified by the research community for various roles. For instance, the nuclease activity of the Cas protein has been disrupted and bound to other proteins or molecules to study genes and non-coding regions (Barrangou and Doudna, 2016). Some roles performed thus far include transcriptional activation or transcriptional repression; imaging achieved by the addition of a fluorophore to the inactivated or "dead" Cas9 protein (dCas9) and epigenetic state alteration by bringing epigenetic repressors or activators to genes.

In 2016, two separate research groups applied CRISPR-Cas9 to *C. neoformans* and *C. deneoformans* research for the first time. Wang et al. (2016) constructed two cassettes containing the gDNA (guide DNA, to be transcribed to gRNA) and *CAS9* nuclease gene, respectively, and made use of electroporation to deliver the cassettes into *C. deneoformans* cells. A human codon optimized *CAS9* nuclease gene, fused to two nuclear localization signals, was employed and placed under the control of a *ACT1* promoter and tailed by a bGHpA terminator. The gDNA was placed under the control of a native U6 gene promoter and 6-T terminator for gRNA production. The cassettes were co-transformed into this yeast and the gRNA was designed to target the *ADE2* gene, creating an adenine auxotroph that forms pink colonies on plates with a low level of adenine. About 82–88% of transformants were pink and sequencing revealed various indel mutations most probably introduced through NHEJ. This group also showed that a single codon change in a targeted gene is possible when a donor DNA cassette is included for HR. Similarly, a hygromycin B resistance marker was introduced into a gene through HR. In the last two instances, the *CAS9* gene and gDNA were both on one vector, allowing co-transformation with the donor DNA.

The real significance of the paper by Wang et al. (2016) was the development of a "suicide" system that got rid of the CRISPR components after a gene has been targeted (**Figure 1**). In this system, the gDNA with or without the addition of *CAS9* are included on the vector with the insert flanked by sites homologous to the targeted gene. After HR, the section of the vector containing the CRISPR components are degraded. Success rates of almost 50% were obtained, even with large fragments containing both the gDNA and *CAS9* gene. Although, HR occurred much more frequently than was seen before, the expression of *CAS9* seemed to diminish the virulence of *C. neoformans* strain H99. This was in contrast with the findings of Arras et al. (2016), the second group to utilize CRISPR-Cas9 in these pathogens. These authors found that *CAS9* expression in *C. neoformans* H99 has no effect on growth, virulence factors or ability to cause disease in a murine inhalation model. The two-step system developed by this group first involved constructing a strain that expresses the *CAS9* gene after integration into a gene-free region of the genome. This is a useful approach which lessens the workload for targeting a series of genes, requiring only the addition of gDNA in subsequent studies. Arras et al. (2016) achieved this *CAS9* integration with biolistic transformation, which yields higher HR rates as was previously seen. Instead of using a RNA polymerase III promoter for gDNA transcription, the authors added two ribozyme genes to the ends of the gDNA. Upon transcription, the fragment undergoes self-cleavage liberating an unaltered gRNA molecule, as described by Gao and Zhao (2014). The advantage of this approach is that any promoter can be used for gDNA transcription. Similar to Wang et al. (2016), Arras et al. (2016) also targeted the *ADE2* gene as proof of concept, adding a successful two-step system to the one-step system of Wang et al. (2016).

A NEW HOPE FOR ELECTROPORATION

Two problems are often encountered when utilizing CRISPR-Cas9: overexpression of the *CAS9* gene that has been shown to be toxic in some fungi, including *S. cerevisiae* and *Schizosaccharomyces pombe,* and off-target mutations that accumulate over time (Wang et al., 2016). The "suicide" CRISPR-Cas9 system developed by Wang et al. (2016) for *C. deneoformans* solves these problems by getting rid of the CRISPR components after the DSB has been made. Before developing this system, they experimented with various methods to get rid of the CRISPR components after gene targeting, which included relying on the tendency of these yeasts to lose extrachromosomal DNA after a few generations, but found that the specific transformants tested remained stable. The "suicide" system also allows for restoration of the disrupted gene for the fulfillment of Falkow's molecular Koch's postulates (Falkow, 1988) and therefore seems like an elegant solution to the challenges faced previously with gene disruption in these yeasts by increasing the occurrence of HR and allowing the use of the less expensive electroporation method for transformation. In contrast, Arras et al. (2016) found that the *CAS9* gene must be integrated into the genome of *C. neoformans* H99 for CRISPR-Cas9 to work in this species. An increase in the frequency of HR was not seen when they co-transformed cells with the gDNA, *CAS9* gene and donor DNA on separate vectors without adding selective pressure to integrate or maintain the CRISPR components, indicating that *C. neoformans* is unable to stably maintain episomal constructs like *C. deneoformans*. Lin et al. (2014), however, obtained 140 stable *C. neoformans* H99 transformants out of 164 total transformants when a G418 resistance gene was used as a selection marker. A transiently expressed system, without *CAS9* genomic integration, could therefore also work in *C. neoformans* if a G418 marker provides selective pressure.

Fan and Lin (2018) recently showed that such a transient system, delivered via electroporation, works well in both *C. neoformans* and *C. deneoformans*. They referred to this system as a TRACE (transient CRISPR-Cas9 coupled with electroporation) system and targeted the *ADE2* gene to evaluate

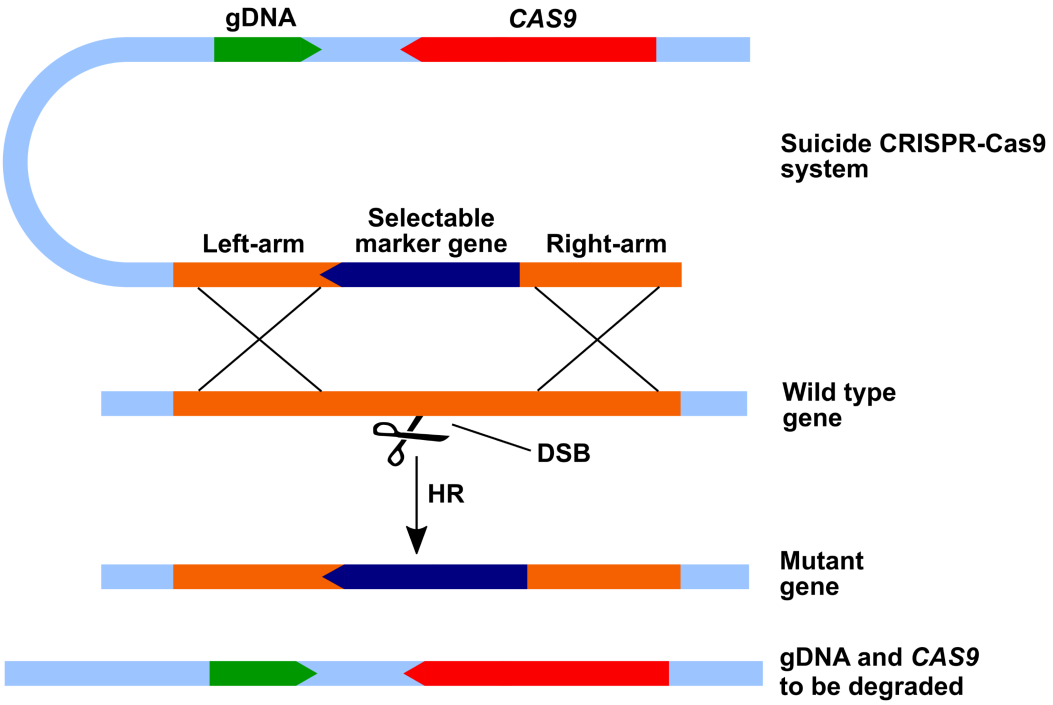

FIGURE 1 | The "suicide" CRISPR-Cas9 system. The CRISPR components are carried on one vector together with a selectable marker flanked by sites homologous to the targeted gene. The Cas9-gRNA causes a double strand break (DSB) after which the selectable marker is introduced into the gene through homologous recombination (HR). The rest of the vector containing the CRISPR components is degraded (adapted from Wang et al., 2016).

effectiveness. The two CRISPR-Cas9 components, *CAS9* gene and gDNA, were placed on separate vectors. The *CAS9* gene was placed under the control of a *GPD1* promoter and terminator while expression of the gDNA was driven by the U6 promoter and a 6-T terminator. A deletion construct containing a nourseothricin resistance gene flanked by arms homologous to the *ADE2* gene was constructed and delivered into cells with electroporation together with the CRISPR-Cas9 components. More than 90% of the *C. neoformans* transformants turned pink, while more than 50% of *C. deneoformans* strain JEC21 and more than 65% of *C. deneoformans* strain XL280 transformants turned pink. Stability assays revealed that most of the transformants retained the pink phenotype while losing the *CAS9* and gDNA vectors since these vectors did not include resistance genes for selection. This group further showed that the rate of gene disruption positively correlates with the dose of *CAS9* and gDNA vectors and that multiple closely related genes can be deleted with one transformation step if the short stretch of gDNA selected allows for targeting of these genes. As proof of concept, the mating type genes, *MFα1-4*, were deleted.

Another laboratory also recently exploited CRISPR-Cas9 technology to improve the practicality of electroporation for gene targeting in *Cryptococcus* species. Wang (2018) described two distinct methods to target *GIB2,* a highly conserved gene in *C. neoformans* and *C. deneoformans*, which encodes an atypical Gβ-like/RACK1 protein. The first technique is similar to the technique described by Fan and Lin (2018), where a transient plasmid carrying CRISPR-Cas9 genes is electroporated into cells for expression *in vivo*. In contrast to the system developed by Fan and Lin (2018), Wang (2018) placed both the *CAS9* gene and gDNA on a single plasmid for electroporation together with the donor DNA. The second technique relied on ribonucleoprotein-mediated CRISPR-Cas9 gene editing. Custom made crRNA for targeting the *GIB2* gene was mixed and incubated at 94°C with universal tracrRNA to facilitate annealing whereafter purified Cas9 protein was added before incubation to allow ribonucleoprotein complex formation. This complex was introduced into cryptococcal cells via electroporation together with donor DNA. Both techniques yielded *GIB2* mutants, although the DNA-based technique seemed to yield more transformants.

CONCLUSION

Even though the biolistic transformation method contributed significantly to what is currently known about *C. neoformans* and *C. deneoformans*, low HR frequencies are still seen in biolistically transformed cells. Low transformation and HR frequencies are often seen in other fungi as well, including other pathogenic fungi. Fu et al. (2006) stated that a high frequency of gene disruption is an exception rather than the norm in fungal pathogens, which complicates functional characterization of genes. Not only is determining the function of genes important for basic research, but a deep understanding of the workings of virulence factors and the genes encoding them is required

to elucidate the mechanism of action of potential drugs. The fundamental challenge to antifungal drug development is the conserved status of many biological processes between humans and fungi, which complicates clinical trial design (Roemer and Krysan, 2014). In addition to this challenge, the low transformation and HR frequency in pathogenic fungi has further slowed the development of novel antifungals in recent times (McCarthy and Walsh, 2017).

The Bio-Rad Biolistic® PDS-1000/He Particle Delivery System is by no means a common sight in a microbiology laboratory and the high cost involved in acquiring such a system and the accompanying consumables have thus far restricted molecular research on *C. neoformans* and *C. deneoformans* to well-resourced research centers. Ironically, the high cost excludes many research laboratories in developing countries, which is also usually the worst affected areas due to immune deficiency caused by the AIDS pandemic. By enabling the use of electroporation, CRISPR-Cas9 technology could therefore bring *Cryptococcus* research right into the midst of the underdeveloped affected areas where researchers have access to more clinical isolates and could supplement the technology currently available to accelerate the discovery of novel drug targets.

Current treatment options include three old and off-patent drugs, amphotericin B (and its liposomal derivatives), 5-fluorocytosine and fluconazole (Perfect and Bicanic, 2015). Due to the high cost and inadequate supply chains, 5-fluorocytosine and amphotericin B drugs frequently do not reach patients in the most affected areas, such as sub-Saharan Africa (Perfect, 2013; Perfect and Bicanic, 2015). This is further made worse by difficulties with monitoring and managing the life-threatening adverse effects of amphotericin B (Perfect and Bicanic, 2015). Fluconazole is, however, donated and distributed by the Pfizer, Inc. Diflucan Partnership Program, which started as an agreement between Pfizer, Inc. and the South African Department of Health and currently provides support to many developing countries across the globe (Wertheimer et al., 2004). Fluconazole is, however, a fungistatic drug and lifelong maintenance therapy is therefore required (Vensel, 2002). This suppressive therapy frequently leads to relapse of this disease in patients in developing countries due to interactions with other drugs, poor compliance with treatment, malabsorption or the development of drug resistance with long term use (Armengou et al., 1996). There is therefore a need for combination therapy to reduce the chance of anti-fungal resistance and to shorten the treatment time (Vanden et al., 1998; Perfect, 2013; Perfect and Bicanic, 2015). No newly developed therapies reached patients in more than 25 years (Perfect, 2017). Treatments currently in the pipeline include APX001; a first-in-class compound that hinders the attachment of adhesion proteins to the outer cell wall, T-2307; an allylamine compound that inhibits the mitochondrial membrane potential and AR-12; a broad-spectrum antifungal for which the specific method of action is still unknown, but probably functions by blocking acetyl-CoA synthetase 1 and by downregulating host chaperone proteins (Perfect, 2017). The development of safer and cheaper treatment options could contribute tremendously to the fight against cryptococcosis, enabled by the new molecular techniques such as the CRISPR-Cas9 gene-targeting tool. Such techniques could prove to be invaluable in studies on the mechanism of action of potential antifungals. CRISPR-Cas9 is therefore not only a valuable healthcare tool that could directly combat human genomic diseases, but is also a valuable search tool in the pursuit of new drug targets in pathogens.

AUTHOR CONTRIBUTIONS

All authors are in agreement with the content of the manuscript. LP conducted the literature study and wrote the draft manuscript. JA, OS, and CP provided inputs, revised, and edited the manuscript.

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Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

SECTION C

The section below forms an additional part of the complete literature review of this study

1. Introduction

Two very simple questions lie at the heart of many scientific investigations: *What is the role of a specific natural phenomenon in its environment?* and *How is this role fulfilled?* In terms of infectious disease, the first question was answered when Louis Pasteur, Robert Koch and others provided proof of the involvement of microorganisms in several diseases (Castillo-Chavez *et al.*, 2016).

In the 1880s, Robert Koch (some sources acknowledge the involvement of others, such as Henle) developed his postulates or set of criteria to establish if a microorganism is the causative agent of a specific disease (Carter, 1985). Koch's postulates stated: (1) A suspected agent must occur in the diseased organism and not in healthy organisms. (2) The causative agent must be shown to be a living organism and must be isolated and obtained in pure form. (3) The agent should cause the same disease when introduced into an otherwise healthy experimental organism. (4) The causative agent should occur in the diseased experimental organism and should be identical to the original agent when isolated and obtained in pure form. The fourth postulate was not part of Koch's original postulates but was later added to confirm causality (Byrd & Segre, 2016).

Two restrictions of these postulates limit the identification and isolation of pathogens, however. The first challenge is the availability of a suitable experimental model organism to infect (Fredericks & Relman, 1996), as some pathogens, such as *Neisseria gonorrhoeae,* only cause disease in humans (Cornelissen *et al*., 1998). Pathogens that only infect humans include most human viruses. A second challenge is the effect of interactions between the microorganism and its environment or other microorganisms on pathogenesis, which further complicates the isolation of the causative agent of a disease. Some pathogens only cause disease in the presence

of other microbes, such as the hepatitis D virus that rely on the hepatitis B virus for some structural components, and some infections only progress to disease state in some hosts while remaining latent in asymptomatic carriers (Fredericks & Relman, 1996). An excellent example of the latter being the case of Typhoid Mary, who was an asymptomatic carrier of typhoid fever (Soper, 1939).

The discovery of nucleic acid and the beginning of molecular biology in the 1950s made it possible to answer the second question: how microorganisms fulfil their role in disease. Since all cellular functions are controlled through genes, a set of criteria was adopted to determine if a gene has any role in the virulence or pathogenicity of a microorganism. To this end, Falkow (1988) modified Koch's postulates to fit the search for virulence genes: (1) The pathogenic phenotype or trait must be present in all pathogenic strains of a species or all pathogenic species of a genus. (2) Specific inactivation of the suspected gene(s) should yield a mutant with a measurable loss in pathogenicity or virulence. (3) Complementation with the functional form of the gene(s) should restore pathogenicity or virulence.

As with Koch's postulates, satisfying the molecular postulates also comes with challenges. Genetic modification of all pathogens is not yet well established, even if a gene was successfully deleted from a genome, restoring a deleted gene to its original form in its original locus is difficult to achieve with current methods (Falkow, 1988). Reintroducing the deleted gene should not disrupt other genes in the process. Arras and co-workers (2015) identified a noncoding region in *Cryptococcus neoformans* into which genes can be integrated for the restoration of a deleted gene. Another challenge occurs when the gene in question is an essential gene, resulting in a non-viable mutant upon deletion. This can be circumvented by cloning the gene into another organism and to look for a change in the phenotype, although this might not work for all genes. Another challenge to these modified postulates is that gene expression is often highly affected by the environment in which the pathogen finds itself and expression could, therefore, vary depending on the host and site of infection. For instance, changes in the gene expression profile are seen when cells move from a low to a high pH (Olson, 1993).

Understanding how microorganisms cause illness is vital in combating infectious disease through vaccines and antimicrobial drugs, especially in a post-antibiotics age which necessitates the identification of new drug targets (Cross, 2008; Jain *et al*., 2010). For instance, 194 genes in *Mycobacterium tuberculosis* are essential for *in vivo* survival in mice, which could all potentially be good targets for tuberculosis drug development (Zhang *et al*., 2006). It makes sense to follow a similar approach to the molecular postulates as with Koch's postulates, to link a gene to a specific virulence trait and ultimately for the identification of potential drug targets, although new developments are needed to mitigate the challenges with genetic modification of most pathogens. Recent developments in genomics and systems biology led to the development of some of these tools needed for the identification of previously unknown targets (Cross, 2008). Using the prokaryotic CRISPR-Cas9 components to modify the genome of the fungal pathogen *C. deneoformans*, formerly known as *C. neoformans* variety *neoformans*, is the focus of this research as an example of such a newly developed tool. The discovery of new drug targets in this global pathogen could significantly enhance the search for novel drugs to better the lives of AIDS patients.

2. Gene targeting

Microorganisms display various traits of interest to humans, some that may be useful in biotechnological processes and others that may contribute to the pathogenicity of pathogens. To determine which genes are responsible for these interesting traits, genes must first be removed from the genome to establish how the trait in question changes. Before nucleotide sequence data and the tools to obtain, modify and study them became widely available, researchers made use of "classical methods" such as exposure to ultra-violet (UV) light and chemical mutagens to introduce mutations into various genes and then screened for cells with alterations in the trait of interest or cells that lacked the trait altogether (Lawrence & Christensen, 1976). Irradiation with UV light was for instance employed to generate *C. neoformans* mutants lacking the ability to form a polysaccharide capsule around the cell wall to determine if the capsule plays a significant role in cryptococcal infections (Bulmer *et al*., 1967). The classical methods are rather laborious and resulted in random mutagenesis as mutations were often introduced in nontargeted locations,

which could affect subsequent studies. These methods also reveal very little about the genes responsible for the studied traits and it was mostly employed to make useful organisms for biotechnological processes and to study how an organism without the specific trait would react in a specific environment. For instance, how a pathogen without a certain characteristic would act when introduced into a host.

When the tools of molecular biology and sequence data became available, techniques relying on the double-strand break (DSB) DNA repair machinery of the cells were employed for mutagenesis. Initially, researchers relied on insertional mutagenesis to randomly insert a sequence into the genome followed by screening for desirable mutants (Labarre *et al*., 1989). It later became possible to study the function of very specific genes by exploiting homologous recombination (HR) (Thomas & Capecchi, 1987). Sequences for insertion are designed with stretches of nucleotides homologous to a gene of interest. When a DSB occurs in the targeted gene, the designed insertion sequence will be integrated into the gene and disrupt gene expression. The inserted sequences were often linked to a genetic marker that could be used to visualise the location of the insertion or to simplify selection of altered cells.

2.1 The homologous recombination pathway in yeast

Apart from playing an important role in chromosome segregation during meiosis, homologous recombination is one of two options available to cells to repair a DSB, with the other being non-homologous end joining (NHEJ) (Huertas, 2010). The HR process has been most extensively studied in *Saccharomyces cerevisiae* but is a fundamental process in all cells and is conserved across groups of related organisms (Aylon & Kupiec 2004; Grenon *et al*., 2006; Huertas, 2010). In the current model described in work by Aylon and Kupiec (2004) as well as Mimitou and Symington (2009), detection of DSBs involves a series of phosphorylation events involving the Tel1 (telomere maintenance 1) and Mec1 (mitosis entry checkpoint 1) kinases; two members of the phosphoinositide-3-kinase-related protein kinase (PIKK) family in yeast. Tel1 associates with the MRX complex consisting of Mre11 (meiotic recombination 11), Rad50 (radiation sensitive 50), and Xrs2 (X-ray sensitive

2) by binding to an interaction motif on the C-terminus on Xrs2. This complex is activated by Tel1 in response to a DSB that cannot be digested (resected) to single-stranded DNA (ssDNA), while the Mec1-MRX complex forms in response to a DSB that can be resected (Grenon *et al*., 2006). Mec1 seems to respond to ssDNA while Tel1 primarily respond to DSBs (Mimitou & Symington, 2009). The exposed double-stranded ends are then resected in the 5' to 3' direction to reveal single-stranded overhangs. This is initially done by the MRX complex and Sae2 (Sporulation in the Absence of Spo11 2), which is activated by Tel1 and Mec1 (Huertas, 2010). MRX and Sae2 have weak exo- and endonuclease activity and can therefore only resect short stretches of terminal dsDNA to reveal 3' ssDNA overhangs (Huertas, 2010). More extensive ssDNA is generated by the nuclease action of either Exo1 (Exodeoxyribonuclease 1) or Sgs1 (ATP-dependent helicase) and Dna2 (ATP-dependent helicase/nuclease). The resulting ssDNA overhangs are then bound by RPA (replication protein A) and later replaced by Rad51 (radiation sensitive 51) with the assistance of accessory factors (Mimitou & Symington, 2009).

The search for a homologous sequence ensues next. During mitosis, this is generally the sister chromatid and the chromosome homolog during meiosis (Mimitou & Symington, 2009). The nucleoprotein filament formed by Rad51 captures and searches a second DNA molecule for homology. This genome-wide search most likely occurs by way of random collisions between the strands resulting in pairing when homology is found (San Filippo *et al*., 2008). The 3'-overhang then acts as a primer for synthesis of a new strand complementary to the homologous donor strand (Mimitou & Symington, 2009). New nucleotides are added to free 3'-hydroxyl groups by DNA polymerase – the involvement of several polymerases is suggested (Harrison & Haber, 2006). The other 3'-overhang is captured by the strand displaced by the first overhang, as predicted in the DSB repair (DSBR) model, and is extended by DNA polymerase. The nicks are sealed with a ligation reaction and a Holliday junction (HJ) forms that can be resolved to yield two separated and sealed double-stranded DNA molecules. The last step is still not well understood in eukaryotes, but Yen1 (Holliday junction resolvase) and Mus81-Mms4 (crossover junction endonucleases) have been shown to be involved (Matos & West, 2014). No joined molecules have been seen in mitotic cells and the synthesis-dependent strand

annealing model has been suggested for recombination in mitotic cells. In this model, the 3'-overhang, that has been extended by DNA synthesis, is displaced and anneals to complementary sequences that are exposed by 5' to 3' resection of the other side of the break, yielding no crossover products (Mimitou & Symington, 2009).

Exploiting HR for gene targeting involves introducing a linearised plasmid, with stretches of DNA homologous to the gene of interest, into cells. An optional reporter gene can be included in between the homologous sites to allow for visualisation of the locus of integration or for the selection of cells in which the gene has been successfully deleted. This plasmid will then serve as a donor DNA for when a DSB occurs in the targeted gene (**Fig. 1**).

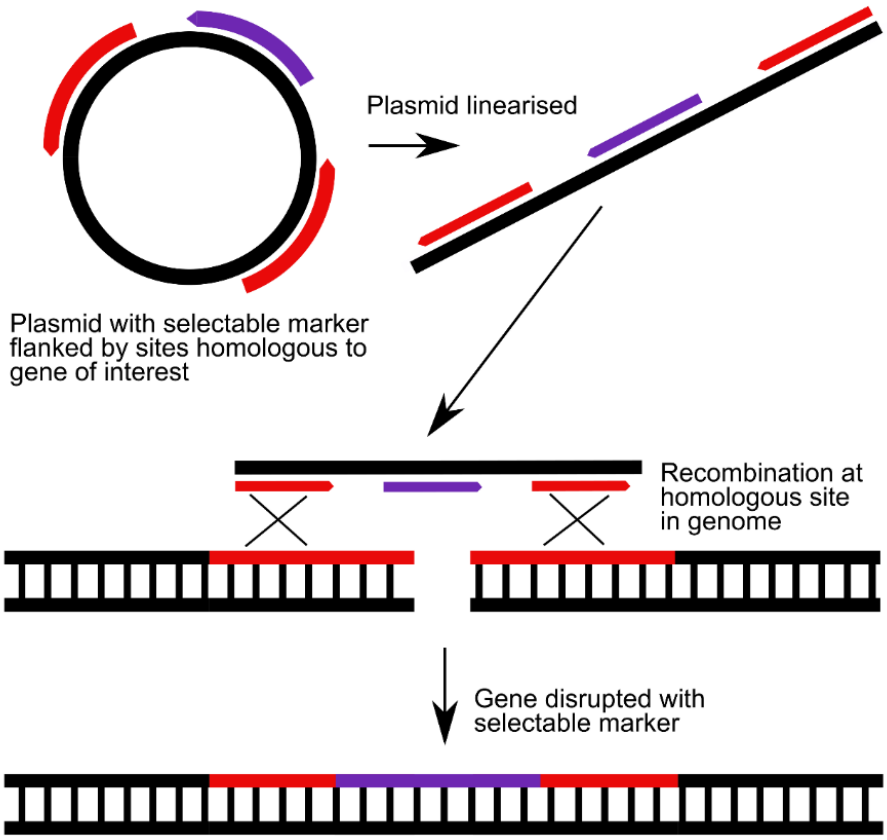

Figure 1: Targeting a gene of interest by introducing a linearised plasmid containing a marker gene flanked with sites homologous to the gene. The marker gene is incorporated into the targeted gene through HR. Adapted from: Aylon and Kupiec, 2004.

2.2 Non-homologous end joining repair in yeast

With this homology-independent DSB repair mechanism, the two separated ends are simply joined together. In the yeast *S. cerevisiae*, three complexes are involved in this repair process; Hdf1/Hdf2 (also known as yKu70/80); Dnl4/Lif1 (DNA ligase 4/ligase-interacting factor 1); and the MRX complex (Mre11/Rad50/Xrs2) (Aylon & Kupiec 2004; Hefferin & Tomkinson, 2005). The two separated ends should first be brought together. If more than one break occurs at the same time, there is a risk that previously unlinked ends may be joined, resulting in genetic instability (Hefferin & Tomkinson, 2005). The Ku yeast homolog complex, Hdf1/Hdf2, is first recruited to the break and binds to each of the exposed ends as a heterodimer. This serves to limit nucleolytic degradation as well as a binding site for end-bridging proteins to bring the ends in juxtaposition (Hefferin & Tomkinson, 2005). The MRX complex binds to the Hdf1/Hdf2 complex and serves as an end joining bridge. The Dnl4 DNA ligase and its co-factor Lif1 is then recruited to the site. This promotes the activity of Rad27 and DNA polymerase beta (Pol4) for processing of the ends and gap filling to generate ligatable ends (Aylon & Kupiec 2004). The ends are then finally connected by Dnl4 to repair the lesion. Processing and gap filling often introduce frame-shift or indel mutations into the repaired DNA which can be exploited for gene targeting.

2.3 Mechanisms to induce a DSB at a specific site for gene targeting

2.3.1 Zinc-finger nucleases (ZFNs)

The most common DNA binding motif in eukaryotes are Cys_2-His_2 zinc-finger domains (Gaj *et al*., 2013). These Cys_2-His_2 zinc-finger domains were first engineered in the 1990s to guide effectors and nucleases to specific DNA targets. Each of these zinc-finger motifs is composed of approximately 30 amino acids, each of which can bind to three nucleotides (Cathomen & Joung, 2008). Assembled zinc-finger arrays, customised to bind to specific DNA sequences, are fused to the nonspecific catalytic domain of the *Fok*I endonuclease (*Flavobacterium okeanokoites* type IIS restriction endonuclease). Since the catalytic domain is only active as a dimer, ZFNs are constructed as two *Fok*I domains each with a zinc tail that recognises a specific sequence (**Fig. 2**). Constructing zinc-finger nucleases have

proven to be laborious, resource intensive, time-consuming, expensive and they suffer from high failure rates (Mahfouz *et al*., 2014).

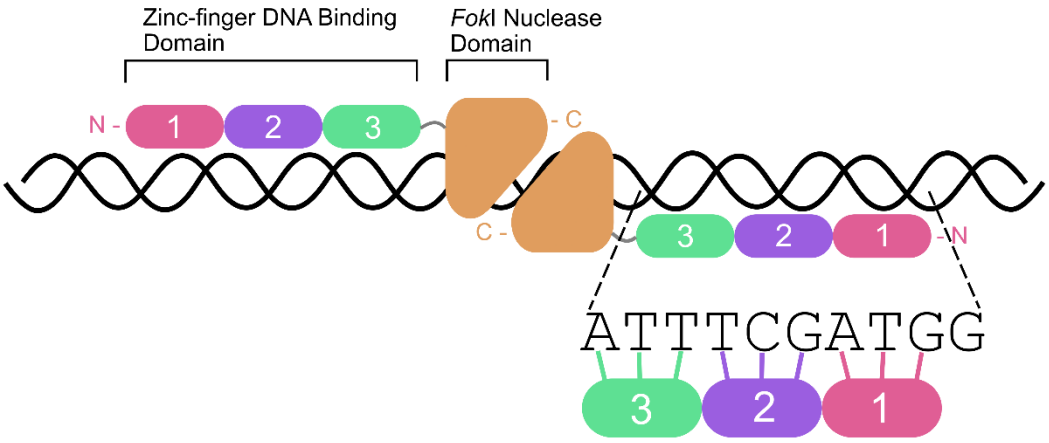

Figure 2: A *Fok*I nuclease dimer with zinc finger tails bound to opposing strands of a double-stranded DNA molecule. Adapted from: Cathomen and Joung, 2008.

2.3.2 Transcription activator-like effector nucleases (TALENs)

TALENs, or transcription activator-like effector nucleases, is a newer technology than zinc-finger nucleases and is slightly easier to construct (Mahfouz *et al*., 2014). Naturally occurring TALEs (occur naturally without nucleases) are utilised by *Xanthomonas* spp., a plant pathogen, to alter DNA transcription in plant host cells to facilitate colonisation (Joung & Sander, 2013). These TALENs are composed of nearly identical 33–35 amino acids-long repeats with hypervariable di-residues at positions 12 and 13 of each repeat. The di-residues dictate the binding specificity to a single nucleotide in the DNA target sequence (**Fig. 3**) To this end, the amino acids histidine and aspartic acid (HD) bind to cytosine (C) nucleotides, asparagine and isoleucine (NI) bind to adenine (A), asparagine and glycine (NG) bind to thymine (T) and the asparagine di-residue (NN) binds to adenine (A) or guanine (G) nucleotides (Joung & Sander, 2013). These DNA binding domains are fused to the same non-specific *Fok*I nuclease domain seen in ZFNs which operate in tandem to cleave DNA. Additional research is, however, required with different repeat domains to determine if other residues bind specific nucleotides with a higher affinity (Joung & Sander, 2013).

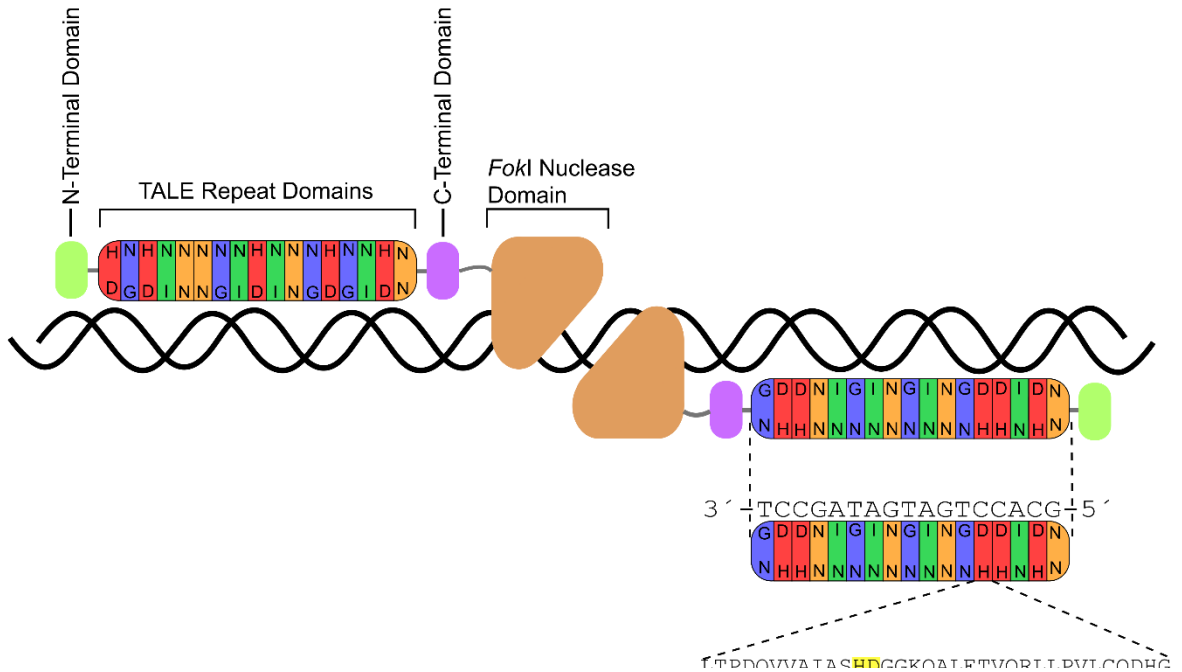

Figure 3: A *Fok*I nuclease dimer with transcription activator-like effector (TALE) domains bound to opposite strands of a double-stranded DNA molecule. Adapted from: Joung and Sander, 2013

2.3.3 CRISPR-Cas9 gene targeting

Using the prokaryotic CRISPR-Cas9 (clustered regularly interspaced short palindromic repeats and CRISPR associated genes) immune system components for gene targeting (**Fig. 4**) is the most recent advancement and was first used for gene targeting in 2012 (Mahfouz *et al*., 2014). Briefly, a short stretch of DNA, complementary to the gene of interest, as well as the *CAS9* gene is introduced into targeted cells (Khandagale & Nadaf, 2016). The complementary sequence, known as the guide DNA (gDNA) sequence, is transcribed into guide RNA (gRNA) and forms a complex with the Cas9 protein. When a sequence complementary to the gRNA is found in the genome of the target cell, a single double-strand break is made by the Cas9 endonuclease in this complementary sequence.

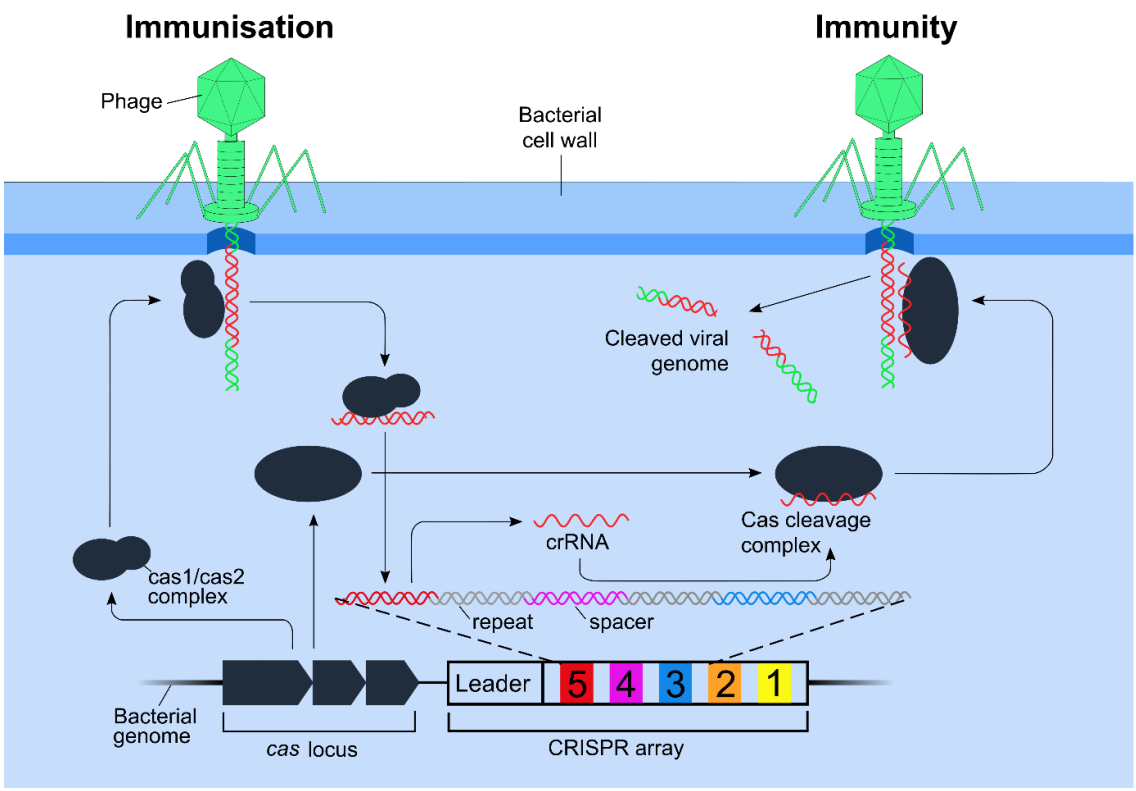

Figure 4: The CRISPR-Cas9 system as an adaptive immune system as seen in many prokaryotes. Adapted from: Ledfort 2017: https://www.nature.com/news/five-big-mysteries-about-crispr-s-origins-1.21294.

3. The pathogenic yeasts *Cryptococcus neoformans* and *C. deneoformans*

3.1 From close siblings to distant relatives

The genus *Cryptococcus* consists of numerous described species with only a handful that are pathogenic to humans. These yeasts are basidiomycetous fungi that cause meningitis; most often in immunocompromised individuals and most notably in patients diagnosed with AIDS (May *et al*., 2016). These pathogens are usually associated with tree bark and bird droppings where spores or desiccated cells, the most likely infectious propagules, are inhaled upon exposure to these environments (Alanio *et al.*, 2015; May *et al*., 2016). When inhaled, the cells localise in the lungs and are engulfed by alveolar macrophages (Alanio *et al.*, 2015). In the absence of an appropriate immune response to the engulfed cryptococcal cells, the cells spread to the central nervous system (CNS), which could lead to potentially fatal meningoencephalitis (May *et al*., 2016).

Cryptococcus neoformans and *C. gattii* are the two species most often encountered in clinical settings, although species such as *C. albidus* and *C. laurentii* have also been implicated in disease (Hajjeh *et al*., 1995). The *C. neoformans*/*C. gattii* species complex originally consisted of the species *C. neoformans* and *C. gattii* and two serotypes were recognised under each species; serotypes A and D under *C. neoformans* and serotypes B and C under *C. gattii* (Kwon-Chung *et al*., 2002). This classification has recently been revised to raise the two *C. neoformans* serotypes to species level as well as to recognise the five molecular types (VGI, VGII, VGIII, VGIIIc and VGIV) of *C. gattii* as separate species (**Table 1**). Debate on this reclassification is however still ongoing (Hagen *et al*., 2015).

Table 1: *Cryptococcus* species as they were previously known with revised names as proposed by Hagen *et al*. (2015)

Previous classification	**Hagen *et al*. (2015) classification**
C. neoformans var. *grubii* (serotype A)	*C. neoformans*
C. neoformans var. *neoformans* (serotype D)	*C. deneoformans*
C. gattii VGI	*C. gattii*
C. gattii VGII	*C. deuterogattii*
C. gattii VGIII	*C. bacillisporus*
C. gattii VGIIIc/VGIV	*C. decagattii*
C. gattii VGIV	*C. tetragattii*

3.2 Classical virulence factors

The most apparent virulence factors in cryptococcal pathogenesis are the ability to grow at 37°C, melanin formation and the thick polysaccharide capsule surrounding pathogenic cryptococcal cells (Esher *et al*., 2018). These virulence factors are well-studied and are often described as the “classical” virulence factors.

The human body temperature is a primary protective factor to protect against infection from pathogens (Perfect, 2006). Like all human pathogens, the pathogenic cryptococcal species have the ability to grow at human body temperature, although the optimal growing temperature is slightly lower at about 32°C (Perfect, 2006). The temperature tolerance between the pathogenic strains and species can vary, for

instance *C. neoformans* have a higher tolerance than *C. deneoformans*. Many genes are important for high-temperature growth, often through examining the transcriptome at various temperatures (Perfect, 2006). Genes involved in stress response, such as oxidative stress, are often also important for growth at high temperatures.

Another classical virulence factor is melanin formation. The copper-containing laccase proteins (Lac1 and Lac2) are involved in the production of melanin by oxidatively polymerising environmental catechols or aminophenols to melanin, which is then deposited into the cell wall (Ikeda *et al*., 2002). Melanin provides protection against physiological stress from its surroundings; its ecological niche as well as from the defence mechanisms of infected hosts. For instance, it acts as a redox buffer against oxidants found in macrophages.

Perhaps the most recognisable virulence trait is the thick capsule surrounding cryptococcal cells. The word “cryptococcus” is derived from the Greek words kryptos (hidden) and kokkos (berry), alluding to the distinctiveness of this trait (Antinori, 2013). The capsule is composed of abundant glucuronoxylomannan (GXM; composed of mannose, xylose and glucuronic acid), and glucuronoxylomannogalactan (GXMGal) as well as mannoproteins (Bielska & May, 2016). Glucuronoxylomannan differs in the degree of xylose addition and acetylation in the different strains (Bose *et al*., 2003). These variations, differentiated by specific antibody binding, contributed to the previous classification of *C. neoformans* and *C. gattii* strains into four serotypes, A through D. The biosynthesis of the capsule components requires many genes (the *CAP* genes) and is well studied, as is the function of the capsule during infection. The capsule acts as a physical barrier against inflammatory cytokines as well as normal macrophage phagocytic function (O'Meara & Alspaugh, 2012).

3.3 Other virulence factors

Many other cellular and physiological traits also play a role during infection. Other cryptococcal virulence factors include prostaglandin production, titan cell formation, mannitol production as well as 3-hydroxy fatty acid production (Chaturvedi, 1996; Erb-Downward & Huffnagle, 2007; Zaragoza & Nielsen, 2013; Madu *et al*., 2017). Most of these virulence traits are involved in stress response and protect against phagocytosis in the lungs. For instance, Madu and co-workers (2017) found that 3-hydroxy fatty acids may potentially diminish the uptake of cryptococcal cells in the lungs, as was observed with amoebae as a model for macrophages. With this amoeba model, it was shown that 3-hydroxy fatty acids may prevent the phagocytosis of cryptococcal cells via suppression of the levels of a fetuin A-like amoebal protein, which may be important for enhancing phagocytosis by amoebae and could likewise also suppress uptake by macrophages in the lungs of an infected host (Madu *et al*., 2017). Cryptococcal cells that produce 3-hydroxy fatty acids are also more resistant to the effects of amoebapore, a hydrolytic enzyme. The 3-hydroxy fatty acid molecules are characterised by the presence of a hydroxyl group at the β-carbon of the fatty acid (Sebolai *et al*., 2008). These fatty acids were previously found to be associated with the cryptococcal capsule and are presumably released into the surroundings through tubular protuberances. These 3-hydroxy fatty acids act as signal molecules in a hormone-like manner across all the domains of life (Sebolai *et al*., 2012). In mammals, these molecules regulate cellular homeostasis and immune responses, while in bacteria and fungi, they may play a role in quorum sensing and biofilm formation. Other effects of these fatty acid molecules on the virulence of cryptococcal yeasts are still unknown. Production of 3-hydroxy fatty acid has been shown to be sensitive to acetylsalicylic acid (Aspirin^{TM}), which is, therefore, a potential drug to lessen the effects of 3-hydroxy fatty acids during cryptococcal infections (Sebolai *et al*., 2008).

4. Purpose of this study

Aim:

To develop an effective CRISPR-Cas9 system for gene deletion in *Cryptococcus deneoformans* that can be used to disrupt virulence genes to study their function and

role during infection. Such a system can potentially be used to determine what effect a drug could have on this pathogen when it interferes with a specific virulence trait, helping in the development of new drugs.

Objectives:

1. To construct plasmid vectors containing the CRISPR-Cas9 components as well as donor DNA fragments (Chapter 2).
2. To transform the plasmid vector carrying the CRISPR-Cas9 components into *C. deneoformans* (Chapter 3).
3. To validate the system by first deleting genes resulting in easily detectable phenotypes (*ade2*Δ and *his3*Δ) (Chapter 3).
4. To use the developed system to delete virulence genes in the *C. deneoformans* mutants (*ade2*Δ and *his3*Δ) obtained in objective 3. Donor DNA will be used to restore the *ade2*Δ and *his3*Δ mutants allowing for easy selection based on altered phenotype. In addition, to compare the virulence mutant phenotypes with the wildtype.

5. References

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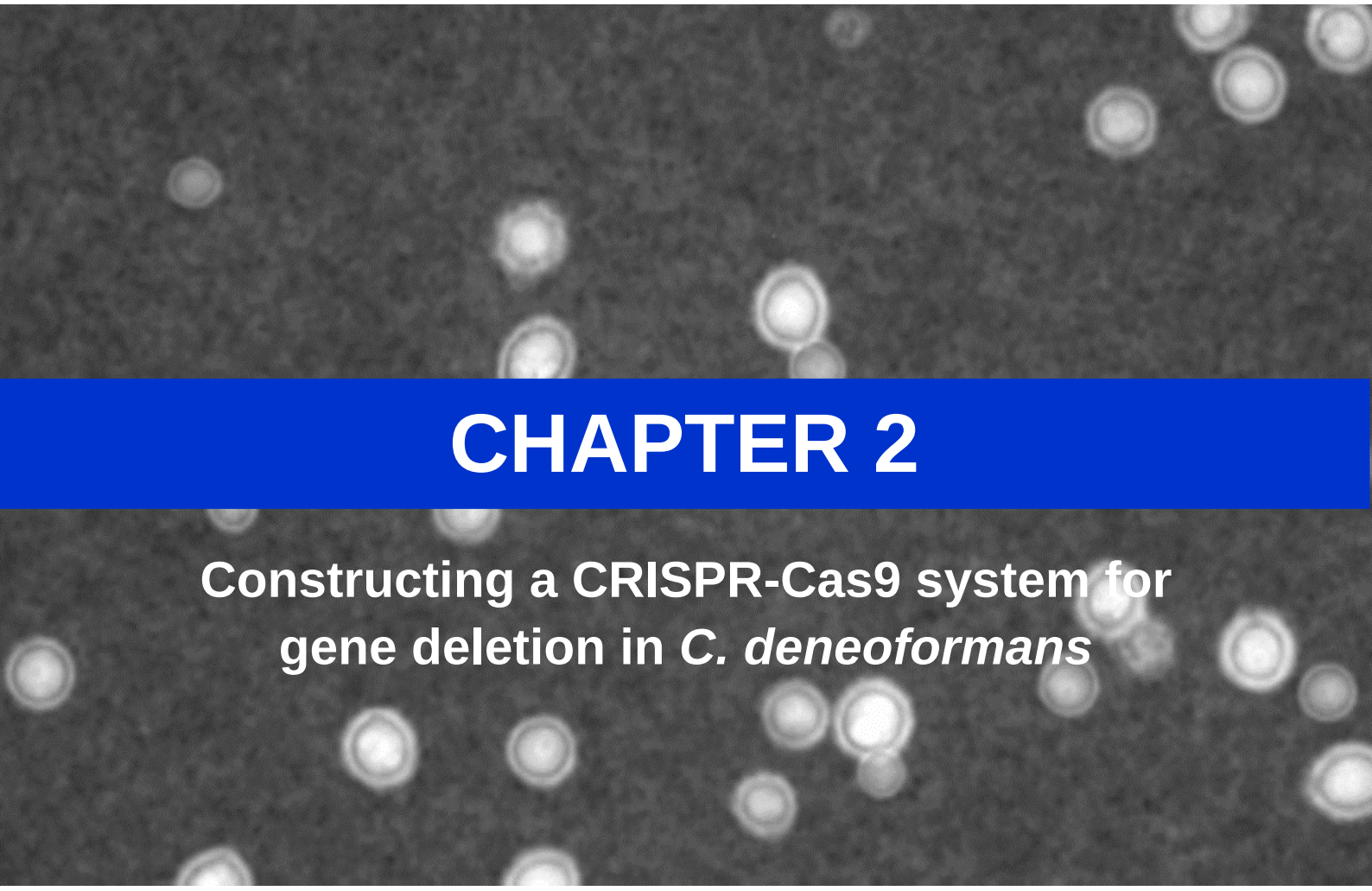

CHAPTER 2

Constructing a CRISPR-Cas9 system for gene deletion in *C. deneoformans*

1. Abstract

The CRISPR-Cas9 genetic machinery – used by bacteria to fend off foreign genetic elements, such as bacteriophages and certain plasmids – have unlocked many doors with regards to genetic engineering and gene targeting. In this study, integrative *CAS9* plasmids, as well as a one-step, transiently-expressed CRISPR-Cas9 system have been employed to target genes in the pathogenic yeast *Cryptococcus deneoformans*. A system consisting of four components have been developed, which include two plasmids carrying both the *CAS9* gene as well as the guide RNA gene, a vector used for the construction of gDNA complementary to 20 bp in a targeted gene, and donor DNA, which is integrated into the cleaved site via homologous recombination. The two plasmids carry different antibiotic resistance markers (conferring resistance to G418 and nourseothricin), which allow for a second round of transformation to restore function to the targeted gene, in line with the principles of Falkow's molecular Koch's postulates.

2. Introduction

The CRISPR-Cas9 gene targeting system has been described as a molecular surgical knife due to its ability to make double-strand breaks (DSBs) at precise locations in the genome (Li & Qian, 2015). As with the use of ZFNs (zinc-finger nucleases) and TALENS (transcription activator-like effector nucleases), this ability of CRISPR-Cas9 gave scientists control over where synthetic donor DNA is integrated in a targeted gene, while they previously had to rely on spontaneous breaks for integration of the donor DNA to disrupt genes (Xiao-Jie *et al*., 2015). The CRISPR-Cas9 system is, however, much easier to design, easier to use and much more effective than other DSB-inducers (DiCarlo *et al*., 2013). This approach to gene targeting also allows for multiplexing, making it possible to edit multiple genes at the same time with a single Cas9 nuclease and individual guide RNA (gRNA) molecules specific to each target (Xiao-Jie *et al*., 2015).

The first use of this system in yeast was in *Saccharomyces cerevisiae* by DiCarlo and co-workers (2013). In their approach, a human codon-optimised *CAS9* gene (*HsCAS9*) was placed under the control of either a *GAL*-L or a *TEF1* promoter and a *CYC1* terminator with an SV40 nuclear localisation signal tagged to the C-terminus of the Cas9 protein. This plasmid was constitutively expressed in yeast cells. The guide DNA (gDNA transcribed to gRNA) was placed under the control of the *SNR52* promoter and *SUP4* terminator on a separate plasmid. The gDNA was designed to target the *CAN1* gene, a plasma membrane arginine permease, in haploid *S. cerevisiae* cells and was delivered into *CAS9* expressing cells together with donor DNA. Up to 78% of cells survived when both CRISPR-Cas9 components were expressed in toxicity assays and a near 100% recombination frequency between the donor DNA and targeted gene was obtained with this system.

In recent years, gene targeting systems also emerged for other yeasts, using the CRISPR-Cas9 system developed by DiCarlo and co-workers (2013) for *S. cerevisiae*. Jacobs and co-workers (2014) developed a system for *Schizosaccharomyces pombe* with a similar *CAS9* expression vector and with gDNA cassettes targeting the *ADE6* gene. Since the ends of the gRNA needs to precisely

defined for proper association with the Cas9 nuclease, the gDNA is usually transcribed by RNA polymerase III (RNA Pol III) with a promoter and terminator for the spliceosomal U6 snRNA that starts transcription in a defined G and terminates in a poly-T stretch (Jacobs *et al*., 2013). This is not possible for all yeast and in the *S. cerevisiae* system, gDNA transcription was placed under the control of a transfer RNA (tRNA) promoter, which results in the addition of a leader sequence (Guffanti *et al*., 2006). The leader sequence is subsequently removed, yielding a gRNA molecule without additional bases at the ends. In *S. pombe*, Jacobs and co-workers (2014) opted for a similar approach. An *RRK1* promoter with a leader sequence was found and added to the gDNA. A hammerhead ribozyme gene was fused to the 3' end of the gDNA to precisely determine the ends of the transcribed gDNA.

Similar CRISPR-Cas9 systems were also developed for a number of other yeasts including, but not limited to *Yarrowia lipolytica* (Schwartz *et al*., 2016), *Komagataella phaffii* (Weninger *et al*., 2016), *Kluyveromyces lactis* (Horwitz *et al*., 2015), *Candida albicans* (Vyas *et* al., 2015), *Cryptococcus neoformans* (Arras *et al*., 2016; Fan & Lin, 2018) as well as *C. deneoformans* (Wang *et al.,* 2016; Fan & Lin, 2018). Wang (2018) developed preassembled CRISPR-Cas9-gRNA ribonucleoproteins that were simply introduced into the *C. neoformans* and *C. deneoformans* cells with electroporation to target genes.

In this chapter, the construction of a CRISPR-Cas9 cassette with both components on a single transiently maintained vector is described for use in *C. deneoformans*. Donor DNA targeting the *ADE2* and *HIS3* genes were also constructed. A second round of transformation to integrate a functional *ADE2* or *HIS3* gene into a virulence gene will restore the wildtype phenotype and give mutants lacking only the targeted virulence gene to limit the effect of other changes in the genome on virulence studies. This system will serve as an in-house system for our laboratory to disrupt and study virulence genes, which is the final objective of this study.

3. Materials and methods

3.1 Maintaining yeast and bacteria strains.

The NEB® (New England Biolabs) 10-beta *Escherichia coli* and XL10-Gold *E. coli* (Agilent Technologies) competent cells for the propagation of plasmids were maintained on lysogeny broth (LB) agar containing 10 $g.L^{-1}$ tryptone, 5 $g.L^{-1}$ yeast extract and 10 $g.L^{-1}$ sodium chloride (NaCl). *Cryptococcus neoformans* H99, the acapsular *C. neoformans* LMPE101 strain and *C. deneoformans* UOFS Y-1378 were maintained on YPD agar (10 $g.L^{-1}$ yeast extract, 20 $g.L^{-1}$ peptone, 20 $g.L^{-1}$ D-glucose, 16 $g.L^{-1}$ agar).

3.2 PCR amplification and primers used

Polymerase chain reaction (PCR) amplification of genes and fragments was a routine procedure during this study. The specific conditions are given where applicable and the names and sequences of the primers used throughout this chapter are provided in **Table 2**.

Table 2: Primers used in chapter 2.

Primer name	Primer sequence
Cn-Gal7-F:pSDMA overlap	GCTGCAGGAATTCGATATCAAGCTTCCAACTTCGCTGACAAGAA
Cn-Gal7-R:pSDMA overlap	CCCCCTCGAGGTCGACAACGAACGATCAGTAGAT
Cd-Gal7-1F_HindIII	CAAGCTTGAGCCATGCATGTATGTTTCC
Cd-Gal7-1R_SalI	CGTCGACGGGTCGATTAAAAGGCGAACA
Cn-25srRNA-1F	AAGTACCGTGAGGGAAAGATGA
Cn-25srRNA-1R	TTGGCTGTGGTTTCGCTAGATA
Cd-GAL7-2F	TAACTCAGTTGTTGTCTCGAAGC
Cd- GAL7-2R	TCTCAAGAGGGGATTGAGCG
Cn-Gal7-2F	AAGTCTGGCGTGAATCCAAT
Cn-Gal7-2R	TCTCAGGAGAGAATTGAGTGCTG
HsCAS9:pSDMA-1F overlap	TTCTCTCCTGAGAATGGACAAGAAGTACTC
HsCAS9:pSDMA-1R overlap	GGATTCACGCCAGACTTTTATCAAACCTTTCTC
Cd-GAL7:HsCAS9-F overlap	CAATCCCCTCTTGAGACAAACGATGGACAAGAAGTACTCCATTG
Cd-GAL7:HsCAS9-R overlap	GACAACAACTGAGTTATTATCAAACCTTTCTCTTTTTCTTAGGGTCC
Cd-Gal7-1F_BamHI	CGGATCCGAGCCATGCATGTATGTTTCC
Safe Haven-2R_AvrII	CCCTAGGAAGAGATGTAGAAACTAGCTTCCTGG
Safe Haven-1F	ATGTTTTCCATCAGTAACATCGGGG
Hammer 1F:cdGAL7 overlap	CGCTCAATCCCCTCTAGAGAAGCACGCTGATGAGTCCGTG
Hammer 1R:cdGAL7 overlap	TCGAGACAACAACTGAGTTAGTCCCATTCGCCATGCCG
Cd-Ade2-CRISPR-1F	ACTGTCGTTTTAGAGCTAGAAATAGCAAGTT
Cd-Ade2-CRISPR-1R	GGAGATCGAGCACGGACGAGCTTAC
pSMA::GAL7p-overlap –F	GCTCTAGAACTAGTGGAGAGCCATGCATGTATGTTTC

pSMA::GAL7p-overlap –R	CCTGCAGCCCGGGGGAGGGTCGATTAAAAGGCGAAC
AHO1099	GTTTTAGAGCTAGAAATAGCAAGTT
Cd CRISPR HIS3-F	CGCTCAATCCCCTCTAGAGAGAGAGACTGATGAGTCCGTGAGGAC GAAACGAGTAAGCT
Cd CRISPR HIS3-R	CTATTTCTAGCTCTAAAACTTGCACTGCAGTTGGAGAGAGACGAGC TTACTCGTTTCGT
Cd-ADE2-1F	GAGTTTTTAGTGATGTTCCTGAG
Cd-ADE2-1R+overlap	GCTATGGTTCCCTGGGTATAATTCAGCATTATG
Cd-ADE2-2F+overlap	TATACCCAGGGAACCATAGCGAGGTATTCCAT
Cd-ADE2-2R	TATTTCTTCAGCCTCTCGATGTA
Cd-HIS3-1F	CCACTCAGCTCACTTGCATTATC
Cd-HIS3-1R+overlap	GTAAAACAGGGTGCAAGAGATATGCGTCT
Cd-HIS3-2F+overlap	TCTCTTGCACCCTGTTTTACCAAAGAGAAGAAA
Cd-HIS3-2R	TTATAAAGCAAGGACACCC

3.3 Genomic extraction of cryptococcal DNA and amplification of *GAL7* and 25S rRNA genes

Cryptococcus neoformans and *C. deneoformans* were grown overnight in 5 mL YPD broth while shaking at 37°C. Approximately 100 mg of wet weight cryptococcal cells were lysed in a bead beater (BeadBug™). A Zymo Research fungal/bacterial DNA miniprep kit was used to extract genomic DNA from these two strains following the manufacturer's instructions.

The *GAL7* gene (encoding galactose-1-phosphate uridyl transferase), including the promoter and terminator regions of about 650 base pair (bp) each, were amplified from the extracted genomic DNA. This was done with a KOD Hot Start DNA Polymerase PCR kit (Novagen) using the primers Cn-Gal7-F:pSDMA overlap (forward) and Cn-Gal7-R:pSDMA overlap (reverse) for *C. neoformans* and primers Cd-Gal7-1F_HindIII (forward) and Cd-Gal7-1R_SalI (reverse) for *C. deneoformans* (see **Table 2**). The primers for *C. deneoformans* included *Sal*I and *Hind*III restriction sites to allow for removal from a cloning vector, while the primers for *C. neoformans* included overlaps to allow for insertion into a plasmid with a NEBuilder® HiFi DNA Assembly kit (NEB®). The conditions used were an initial denaturation step of 2 min at 94°C, 25 cycles of a denaturing step of 20 sec at 95°C, annealing step of 10 sec at 56°C (*C. deneoformans*) or 60°C (*C. neoformans*) and an elongation step of 2 min 30 sec at 70°C. The 25 cycles were followed by a final elongation step of 7 min at 72°C. The PCR products were about 2700 bp in size and were visualised with gel electrophoresis to confirm successful amplification. Briefly, this involved loading the

DNA samples into the wells of a solidified 0.8% (w/v) gel prepared by dissolving agarose in TAE electrophoresis buffer. A 10 kb O'GeneRuler DNA Ladder (Thermo Fisher Scientific) was added for size comparison and ethidium bromide or SYBR Safe DNA Stain (Thermo Fisher Scientific) was added to the unsolidified gel to allow for visualisation under UV light in a Gel Doc™ XR+ (Bio-Rad). The gel was exposed to an electrical current at 90 volts for about 30 min before visualisation. The same method was also followed to validate if the correct insert was inserted into the vectors after digestion in the subsequent steps.

A 2000 bp region in the 25S rRNA gene of *C. deneoformans* was also amplified using the same PCR program as described earlier, except with an annealing temperature of 60°C. The primers Cn-25srRNA-1F (forward) and Cn-25srRNA-1R (reverse) were used.

3.4 *HsCAS9* plasmids

3.4.1 Adding the *GAL7* promoter and terminator to pSDMA plasmids

The *GAL7* gene amplified from *C. deneoformans* genomic DNA was cloned into a pMiniT 2.0 cloning vector (NEB®) following the manufacturer's instructions. The resulting plasmid was extracted from XL-10 Gold *E. coli* competent cells using a Sigma-Aldrich GenElute™ Plasmid Miniprep kit, following the manufacturer's instructions. These cells were plated on LB agar plates containing 100 $mg.L^{-1}$ ampicillin (Roche®) for selection and cultivated overnight at 37°C while shaking. Before extraction with the kit, colonies were screened for correct inserts by following a plasmid extraction protocol described by Sambrook and co-workers (1989) and expected bands were confirmed with a diagnostic restriction digest. This procedure was followed after every plasmid propagation step in this study. The extracted plasmids were digested with *Sal*I and *Hind*III restriction enzymes (Thermo Fisher Scientific) to remove the *GAL7* gene from the NEB® pMiniT 2.0 vector. The digest reaction was analysed on a gel and the *GAL7* fragment excised from the gel and cleaned with a Qiagen QIAquick Gel Extraction kit, following the instructions provided. The *GAL7* gene was then ligated into similarly digested and purified

pSDMA25 and pSDMA58 plasmids obtained from Addgene (Arras *et al*., 2015) following the instructions provided by the manufacturer of the T4 DNA Ligase kit (Thermo Fisher Scientific). The resulting plasmids were extracted from XL-10 Gold *E. coli* cells using the Sigma-Aldrich GenElute™ Plasmid Miniprep kit as previously described.

The *GAL7* gene amplified from *C. neoformans* genomic DNA with overlap primers was cloned into pSDMA25 and pSDMA58 plasmids linearised with *Sal*I and *Hind*III restriction enzymes. Cloning was done with the NEBuilder® HiFi DNA Assembly kit (NEB®). The resulting plasmids were propagated in and then extracted from NEB® 10-beta *E. coli* cells using the Sigma-Aldrich GenElute™ Plasmid Miniprep kit.

The *GAL7* open reading frame (ORF) was removed from the pSDMA25 and pSDMA58 plasmids containing the *C. neoformans* or *C. deneoformans GAL7* genes by PCR amplifying the region around the ORF. The same PCR program as previously mentioned was used with annealing temperatures of 55°C to remove the *C. deneoformans* ORF and 50°C to remove the *C. neoformans* ORF. The primers Cd-GAL7-2F (forward) and Cd- GAL7-2R (reverse) were used for *C. deneoformans* and the primers Cn-Gal7-2F (forward) and Cn-Gal7-2R (reverse) were used to remove the *C. neoformans GAL7* ORF.

3.4.2 Inserting the *HsCAS9* gene in between the *GAL7* promoter and terminator

The human codon-optimised *CAS9* gene was PCR amplified from the pAO815 plasmid obtained from GenScript. The same PCR program described earlier was used with an annealing temperature of 58°C and the following primer pairs were used for amplification: HsCAS9:pSDMA-1F overlap (forward) and HsCAS9:pSDMA-1R overlap (reverse) for *C. neoformans* and Cd-GAL7:HsCAS9-F overlap (forward) and Cd-GAL7:HsCAS9-R overlap (reverse) for *C. deneoformans*. The PCR product was digested with *Dpn*I (Thermo Fisher Scientific) to remove the plasmid template. A

NEBuilder® HiFi DNA Assembly kit (NEB®) was used to clone the amplified *HsCAS9* gene into each of the amplified pSDMA plasmids in between the *GAL7* promoter and terminator, in place of the *GAL7* ORF. This created the plasmids pCd100 (*C. deneoformans* Gal7::pSDMA58 (HYG)::HsCAS9) and pCd102 (*C. deneoformans* Gal7::pSDMA25 (NAT)::HsCAS9), which were used later on in the study. A list of all the plasmids constructed throughout this study for *C. deneoformans* can be found at the end of the results and discussion section of this chapter (**Table 3**).

3.4.3 Replacing the Safe Haven region with the *C. deneoformans* 25S rRNA gene

The pSDMA plasmid vectors contain a Safe Haven region to allow for integration in *C. neoformans*. This region was replaced in the vectors for *C. deneoformans* integration with a 2000 bp region from the 25S rRNA gene. The 25S rRNA region (amplified in section 3.3) were ligated into the NEB® pMiniT 2.0 vector, following the manufacturer's instructions. The resulting plasmid was extracted from NEB® 10-beta *E. coli* competent cells as before. The *GAL7* promoter region, *HsCAS9* gene, *GAL7* terminator region and hygromycin (pSDMA58) or nourseothricin (pSDMA25) resistance markers were PCR amplified using the same program as described before with an annealing temperature of 56°C. The following primer pair was used: Cd-Gal7-1F_BamHI (forward) and Safe Haven-2R_AvrII (reverse) resulting in a product of about 7500 bp. This product was then cloned into the NEB® pMiniT 2.0 vector and the resulting plasmid was extracted as before. The selectable marker region and *GAL7* promoter and terminator region enclosing the *HsCAS9* gene were removed from the NEB® pMiniT 2.0 vectors by digesting with *Avr*II and *BamH*I and removed and cleaned from a gel as described earlier. The 25S rRNA vector was linearised with *Avr*II and *Bgl*II and the *HsCAS9* region with expression components and marker were cloned into the 25S rRNA vector using a T4 DNA Ligase kit (Thermo Fisher Scientific) and extracted as previously described.

3.5 Transient plasmids for *C. deneoformans*

3.5.1 Removal of Safe Haven region from the pSDMA25 plasmid

To construct plasmids for transient expression of the CRISPR components in *C. deneoformans*, the Safe Haven region was removed to reduce the size of the plasmids. Part of this region was removed by PCR amplifying the region around the Safe Haven site with primer pair Safe Haven-1F (forward) and Safe Haven-2R_AvrII (reverse). An Expand™ Long Template PCR System (Roche®) was used with the following conditions: initial denaturation at 94°C for 2 min, 15 cycles of denaturation at 94°C for 10 sec, annealing at 59°C for 30 sec and elongation at 68°C for 6 min. This was followed by an additional elongation step of 68°C for 7 min. This product was cloned into the NEB® pMiniT 2.0 vector and digested with *Avr*II to remove the rest of the Safe Haven site. The resulting linear plasmid was ligated using the T4 DNA Ligase kit (Thermo Fisher Scientific) to obtain a circular plasmid, transformed into XL-10 Gold competent *E. coli* cells and extracted using a Thermo Fisher Scientific GeneJET Plasmid Miniprep Kit following the manufacturer's instructions. The modified pSDMA25 plasmid is now known as pCd103.

3.5.2 Cloning the *HsCAS9* gene into a the pSDMA57 plasmid

A third pSDMA plasmid, pSDMA57, was obtained from Addgene to add another selection marker to the system. This plasmid has a neomycin resistance marker that also confers resistance to geneticin (G418). The Safe Haven region was removed from this plasmid in a similar manner as described in section 3.5.1. The *HsCAS9* gene region flanked by the *GAL7* promoter and terminator was removed from the pCd102 plasmid by digesting with *Sac*II and *Sal*I. The pSDMA57 plasmid was digested in the same manner. The fragments were cleaned using a Qiagen QIAquick Gel Extraction kit and the *HsCAS9* region was ligated into the pSDMA57 plasmid using the T4 DNA Ligase kit as before. Ligated plasmid was transformed into XL-10 Gold competent *E. coli* cells and extracted as previously described. This yielded the pCd104 plasmid.

3.5.3 Removing the *C. deneoformans GAL7* ORF in pMiniT2.0::CdGAL7

The NEB® pMiniT 2.0 vector containing the *C. deneoformans GAL7* gene prepared in section 3.4.1 was PCR amplified to remove the *GAL7* ORF. The same program for the KOD Hot Start Polymerase (Novagen) was used as described earlier. An annealing temperature of 57°C was used and the following primer pair was added to the reaction: Cd-Gal7-2F (forward) and Cd-Gal7-2R (reverse).

3.5.4 Adding the hammerhead and hepatitis delta virus ribozyme genes into the pMiniT 2.0 vector without the *C. deneoformans GAL7* ORF

The plasmid pRS316-RGR-GFP was obtained from Addgene and the hammerhead ribozyme gene, green fluorescence protein guide DNA (GFP gDNA) and hepatitis delta virus (HDV) ribozyme gene were PCR amplified from this plasmid with overlap primers for insertion into pCd105. The program for KOD Hot Start Polymerase (Novagen) was used with an annealing temperature of 60°C. The primer pair Hammer 1F:cdGAL7 overlap (forward) and Hammer 1R:cdGAL7 overlap (reverse) was used for amplification. The obtained PCR product as well as the PCR amplified pCd105 plasmid were digested with *Dpn*I to remove the PCR template. The ribozyme and gDNA fragment was then cloned into the pCd105 plasmid using the NEBuilder® HiFi DNA Assembly kit (NEB®). Plasmids were transformed into NEB® 10-beta *E. coli* competent cells as described before and extracted after propagation using a GeneJET Plasmid Miniprep kit (Thermo Fisher Scientific). Restriction digest with *Eco*RI was done to determine if insertion was successful. This plasmid without the *GAL7* ORF and the ribozyme genes is known as pCd105.

3.5.5 Inserting the *C. deneofromans ADE2* gDNA into pCd105

To replace the GFP gDNA site with gDNA targeting the *C. deneoformans ADE2* (encoding phosphoribosylaminoimidazole carboxylase) gene, the construct around the GFP gDNA was PCR amplified with primers with overhangs to insert the short *ADE2* gDNA target site into pCd105. The KOD Hot Start Polymerase program was once again utilised for this purpose with an annealing temperature of 57°C. The primer pair Cd-Ade2-CRISPR-1F (forward) and Cd-Ade2-CRISPR-1R (reverse) was

used. The resulting PCR product was analysed on an agarose gel and purified with a Qiagen QIAquick Gel Extraction kit, following the instructions provided. The purified fragment was then phosphorylated to allow for recirculation of the linear construct using a T4 polynucleotide kinase kit (Thermo Fisher Scientific). The phosphorylated fragment was ligated and transformed into XL-10 Gold *E. coli* competent cells and extracted after overnight cultivation using a GeneJET Plasmid Miniprep kit (Thermo Fisher Scientific) as previously described. The resulting plasmid was labelled pCd106.

3.5.6 Inserting the *ADE2* gDNA construct from pCd106 into pCd103 and pCd104

The *C. deneoformans ADE2* gDNA construct, including the *GAL7* promoter and terminator, the ribozymes and the *ADE2* gDNA and target site, was PCR amplified using a KAPA Taq ReadyMix PCR kit (KAPA Biosystems) and pCd106 as template. The program was as follows: initial denaturation for 3 min at 95°C; 30 cycles including a denaturation for 20 sec at 98°C; annealing for 15 sec at 65°C and elongation for 90 sec at 72°C. The final elongation step was kept at 72°C for 90 sec. The overlap primers used were pSMA::GAL7p-overlap –F (forward) and pSMA::GAL7p-overlap –R (reverse). The PCR product was run on a gel and purified using a GeneJET Gel Extraction kit (Thermo Fisher Scientific) and following the manufacturer's instructions.

The pCd103 and pCd104 plasmids were linearised by digestion with *Bam*HI (Thermo Fisher Scientific) and analysed on an agarose gel before being purified using a GeneJET Gel Extraction kit (Thermo Fisher Scientific). A NEBuilder® HiFi DNA Assembly kit (NEB®) was then used to clone the PCR product into the linearised pCd103 and pCd104 plasmids. Reaction mixture was used to transform NEB® 10-beta *E. coli* competent cells. The cells were incubated overnight, screening was done, and plasmids were extracted using a GeneJET Plasmid Miniprep kit (Thermo Fisher Scientific). The resulting plasmids were labelled pCd107 (with a nourseothricin resistance marker) and pCd108 (with a neomycin resistance marker).

3.5.7 Constructing and inserting *C. deneoformans HIS3* gDNA into pCd106

The *ADE2* gDNA region in plasmid pCd106 was PCR amplified to linearise the plasmid and to remove the *ADE2* CRISPR site. This was done with the Expand™ Long Template PCR System (Roche®) kit, as described before. An annealing step of 30 sec at 50°C repeated for 30 cycles was employed. The primer pair AHO1099 (forward) and Cd-Gal7-2R (reverse) was used. *Dpn*I restriction enzyme was added to the completed PCR to remove the template. The PCR product was analysed on an agarose gel and the resulting bands were purified using a GeneJET Gel Extraction kit (Thermo Fisher Scientific).

To construct the *C. deneoformans HIS3* (encoding Imidazoleglycerol-phosphate dehydratase) gDNA and CRISPR site, an oligonucleotide pair was polymerised with PCR to obtain a construct of 101 bp. The pair, Cd CRISPR HIS3-F (forward) and Cd CRISPR HIS3-R (reverse) were polymerised using the KAPA Taq ReadyMix PCR kit (KAPA Biosystems) as described before, with an annealing temperature of 70°C for 15 sec and repeated for 30 cycles. The resulting fragment was visualised on a 2% (w/v) low melting point agarose gel. The bands were excised from the gel and purified using a GeneJET Gel Extraction kit (Thermo Fisher Scientific), following the instructions provided. The constructed *HIS3* gDNA was cloned in place of the *ADE2* gDNA in the linearised pCd106 plasmid from which the *ADE2* gDNA has been removed. Cloning was done using a NEBuilder® HiFi DNA Assembly kit (NEB®). Transformation of NEB® 10-beta *E. coli* competent cells followed and after overnight cultivation and screening, the plasmid was extracted using the GeneJET Plasmid Miniprep kit (Thermo Fisher Scientific), following the instructions provided. This plasmid was labelled as pCd109.

3.5.8 Inserting the *C. deneoformans HIS3* gDNA from pCd109 into pCd103 and pCd104

The *C. deneoformans HIS3* gDNA together with the *GAL7* promoter and terminator and the ribozymes were PCR amplified from the pCd109 plasmid with overlap primers, pSMA::GAL7p-overlap –F (forward) and (pSMA::GAL7p-overlap –

R). A KAPA Taq ReadyMix PCR kit (KAPA Biosystems) was used for this purpose and an annealing step of 65°C for 15 sec repeated for 30 cycles was used. The resulting bands were visualised on a 0.8% (w/v) agarose gel and extracted and purified using a GeneJET Gel Extraction kit (Thermo Fisher Scientific). These purified fragments were then cloned into the pCd103 and pCd104 plasmids linearised with *BamH*I in section 3.5.6 using a NEBuilder® HiFi DNA Assembly kit (NEB®) as described earlier. Transformation into NEB® 10-beta *E. coli* competent cells then followed. After overnight cultivation, the cells were screened for the correct insert and plasmid were extracted following the instructions provided by the GeneJET Plasmid Miniprep kit (Thermo Fisher Scientific). These plasmids were labelled as pCd110 and pCd111.

3.6 Constructing *C. deneoformans ADE2* and *HIS3* donor DNA

Short linear fragments were constructed to serve as donor DNA for homologous integration (HR) when targeting the *C. deneoformans ADE2* and *HIS3* genes. This was done with cloning-free stitching PCR. Two oligonucleotides were polymerised with PCR with each having a complementary overlap to the other. A subsequent PCR polymerisation joined the two oligonucleotides together to yield a completed donor DNA fragment. For *ADE2*, the oligonucleotide pair Cd-ADE2-1F (forward) and Cd-ADE2-1R+overlap (reverse) were used to make the first half of the donor DNA, while the pair Cd-ADE2-2F+overlap (forward) and Cd-ADE2-2R (reverse) was used to construct the second half. Polymerisation was done with a KAPA Taq ReadyMix PCR kit (KAPA Biosystems) employing an annealing temperature of 60°C maintained for 15 sec for 30 cycles. The same program was also used to assemble the *HIS3* donor DNA, using the oligonucleotide pair Cd-HIS3-1F (forward) and Cd-HIS3-1R+overlap (reverse) for the first half and pair Cd-HIS3-2F+overlap (forward) and Cd-HIS3-2R (reverse) for the second half. These fragments were analysed on a gel and purified using the GeneJET Gel Extraction kit (Thermo Fisher Scientific). The two halves of each of the two donor DNA fragments were added together and polymerised with PCR using the same program and kit as before. The resulting completed donor DNA fragments were analysed on a gel and purified as before to be transformed into *C. deneoformans*. The *ADE2* donor DNA is 436 bp in length while

the *HIS3* fragment is 308 bp in length. A NanoDrop™ microvolume spectrophotometer (Thermo Fisher Scientific) was used to determine the concentration of the donor DNA before transformation.

4. Results and discussion

4.1 Construction of *HsCAS9* plasmids for *C. neoformans* and *C. deneoformans*

The first attempt at developing a CRISPR-Cas9 system for *C. deneoformans* involved the construction of a plasmid carrying the *HsCAS9* gene. For this purpose, plasmids pSDMA25 and pSDMA58, developed by Arras and co-workers (2015), were obtained from Addgene (www.addgene.com) a non-profit plasmid depository. These plasmids contain a region homologous to a genomic "Safe Haven" region on chromosome 1 in *C. neoformans*. This intergenic, non-coding region is 1544 bp in length and allows for integration of these plasmids to obtain stable transformants. Furthermore, the pSDMA25 and pSDMA58 plasmids confer resistance to nourseothricin and hygromycin B, respectively. The plasmids were linearised using *Hind*III and *Sal*I in a restriction digest reaction (**Fig. 5**). Genomic DNA was extracted from *C. neoformans* and *C. deneoformans* and the *GAL7* genes from each of these species were PCR amplified (**Fig. 5 and 6**). The amplified *GAL7* genes were approximately 2700 bp in length. The *C. deneoformans* gene was first cloned into a cloning vector to allow for restriction digestion at the ends of the gene. This gene was then ligated into each of the linearised pSDMA plasmids. The *C. neoformans GAL7* gene was amplified with overlap primers to add regions complementary to the ends of the linearised pSDMA plasmids for integration into these plasmids.

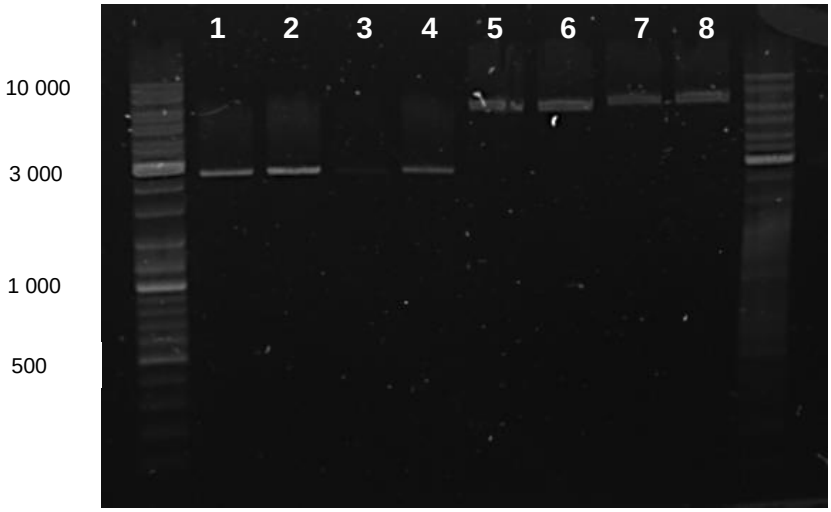

Figure 5: Lanes 1 to 4 show the PCR amplified *C. neoformans GAL7* gene, while lanes 5 and 6 show the linearised pSDMA25 plasmid and lanes 7 and 8 the pSDMA58 plasmid. A 10 kb O'GeneRuler^{TM} DNA Ladder (Thermo Fisher Scientific) was used for size comparison.

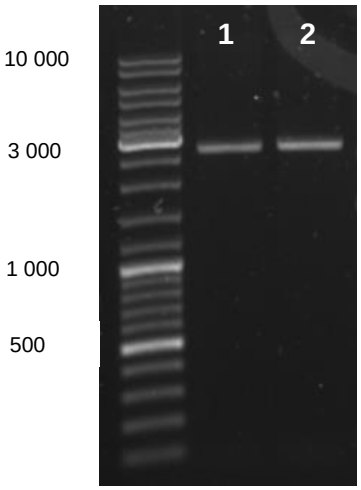

Figure 6: Lanes 1 and 2 show the PCR amplified *C. deneoformans GAL7* gene.

The cryptococcal *GAL7* gene encodes galactose-1-phosphate uridyl transferase, a protein that plays a role in the conversion of galactose to glucose-6-phosphate (Wickes & Edman, 1995). The promoter of this gene has been identified by Wickes and Edman (1995) as an inducible promoter for the expression of genes only when cells are cultivated on galactose as carbon source in the absence of glucose. The *GAL7* ORF was removed by PCR amplifying the region around the ORF to obtain the *GAL7* promoter and terminator (**Fig. 7**).

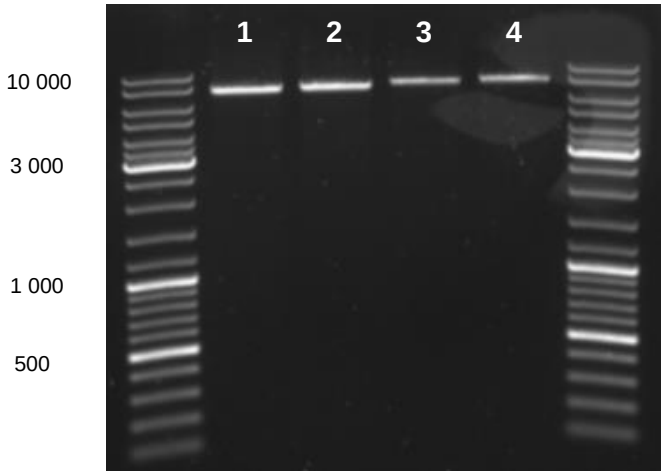

Figure 7: Lanes 1 and 2 show the PCR amplified pSDMA25 plasmid containing the *C. neoformans GAL7* gene to remove the *GAL7* ORF. Lanes 3 and 4 show the same for the pSDMA58 plasmid.

A human codon-optimised *CAS9* gene was found to function well in *C. deneoformans* and *C. neoformans* when used by others and was therefore also the gene of choice for this study (Arras *et al*., 2016; Wang *et al*., 2016). This gene was PCR amplified using overlap primers with bases complementary to the bases at the ends of the PCR-linearised pSDMA plasmids. The source of this gene was the pAO815 plasmid obtained from GenScript and is about 4100 bp in length (**Fig. 8**). The *HsCAS9* gene was inserted between the *GAL7* promoter and terminator in each plasmid. Successful integration was confirmed with *Bam*HI restriction digest (**Fig. 9 and 10**). The plasmids prepared for *C. deneoformans* were labelled as pCd100 (pSDMA58) and pCd102 (pSDMA25) (**Fig. 13 and 14**).

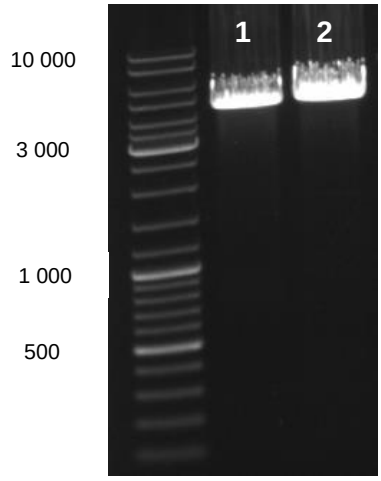

Figure 8: Lanes 1 and 2 show the *HsCAS9* gene PCR amplified with overlap primers for integration between the *C. deneoformans GAL7* promoter and terminator.

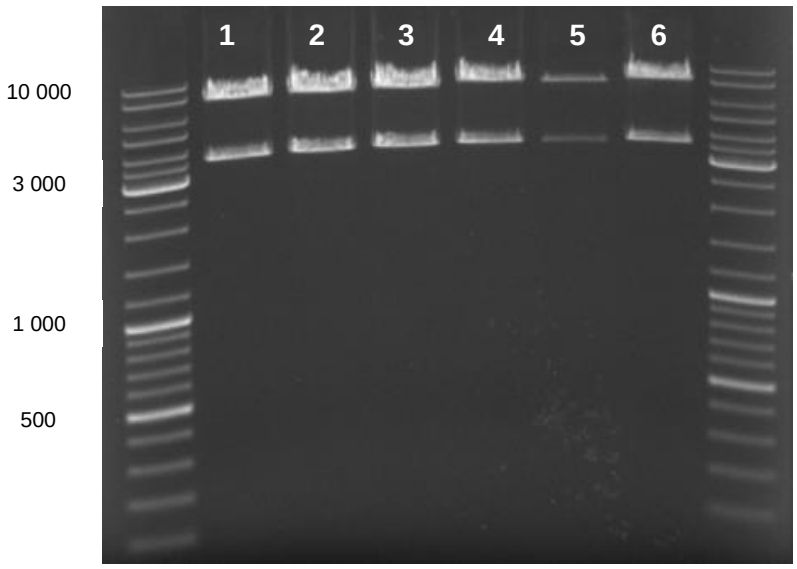

Figure 9: Lanes 1 to 3 show the pCd102 plasmid digested with *Bam*HI, while lanes 4 to 6 show the pCd100 plasmid, digested with the same restriction enzyme.

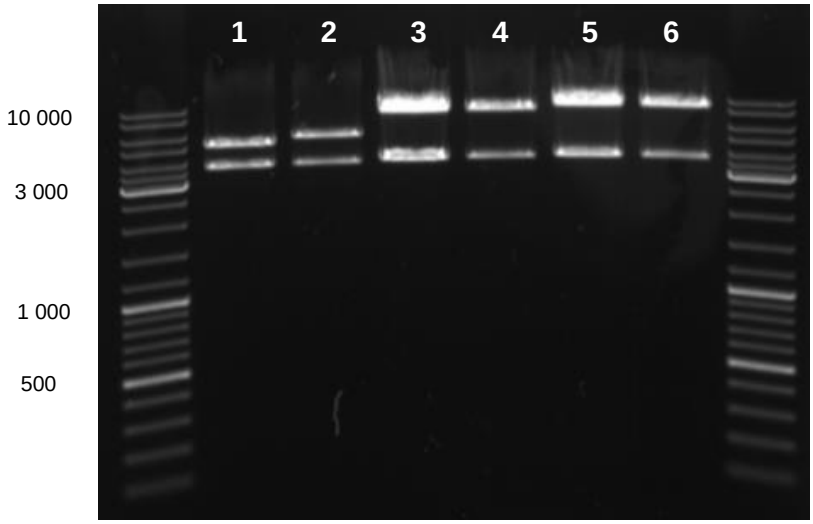

Figure 10: Lane 1 shows the pSDMA25 plasmid without the *HsCAS9* gene in place of the *C. neoformans GAL7* ORF, while lane 2 show the same for the pSDMA58 plasmid. Lanes 3 to 4 show the pSDMA25 plasmid with *C. neoformans GAL7* promoter, terminator and the *HsCAS9* gene. Lanes 5 to 6 show the same for the pSDMA58 plasmid. All of these plasmids were digested with *Bam*HI.

Since the Safe Haven site in the pSDMA plasmids was homologous to the genome of *C. neoformans* and not *C. deneoformans*, it was decided to replace this site with a ~2000 bp region homologous to the 25S rRNA gene in *C. deneoformans* for integration into the genome of this species. No interference with the normal functioning of this gene would occur as multiple copies of the 25S rRNA gene are found in the genome. The PCR amplified 25S rRNA gene can be seen in **Fig. 11** and the completed (linearised) 25S rRNA plasmids with the *C. deneoformans GAL7* promoter, terminator and *HsCAS9* gene is shown in **Fig. 12**.

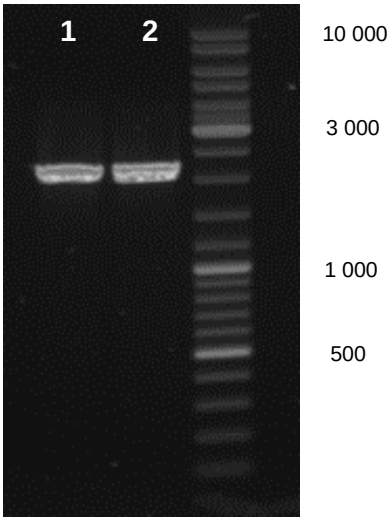

Figure 11: Lanes 1 and 2 show the PCR amplified 25S rRNA gene of *C. deneoformans.*

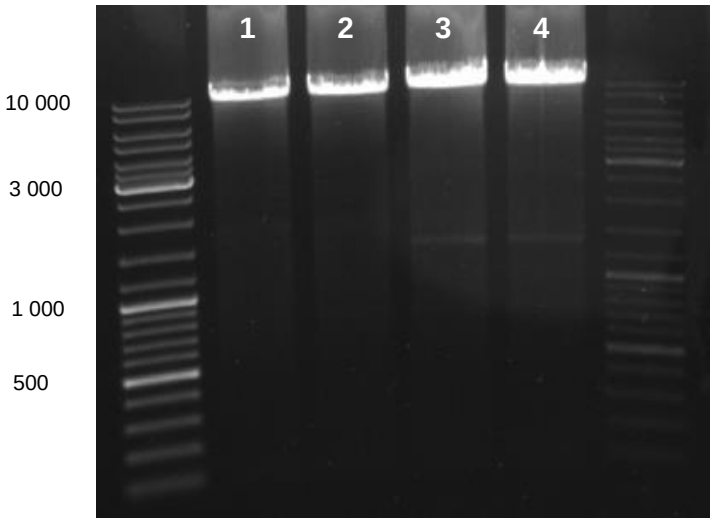

Figure 12: Lanes 1 and 2 show the 25S rRNA plasmid containing the nourseothricin resistance marker, while lanes 3 and 4 show the plasmid containing the hygromycin B resistance marker.

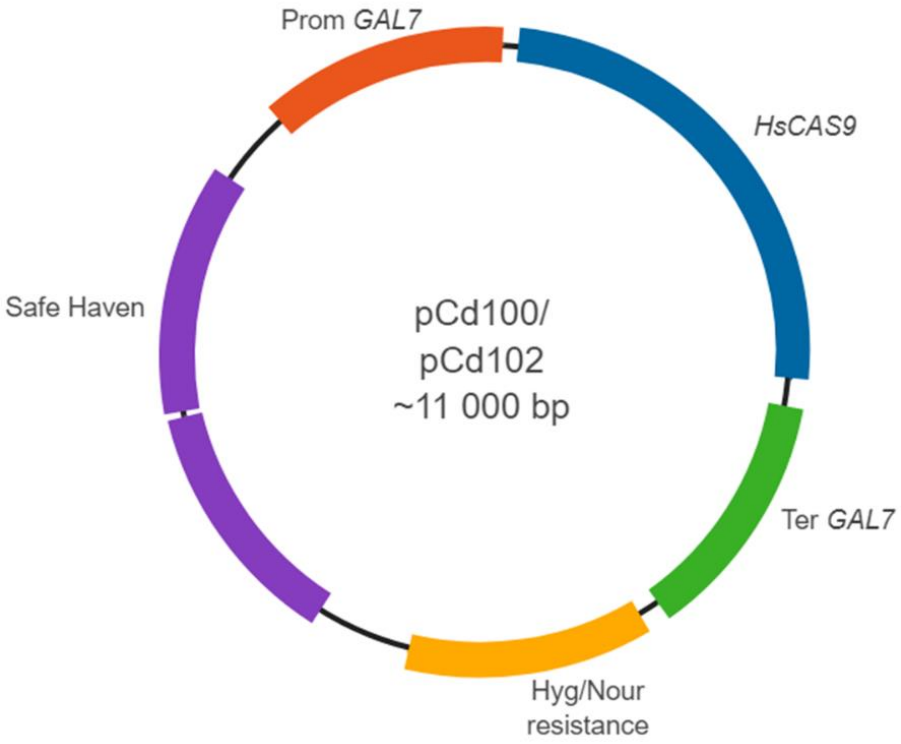

Figure 13: A simplified vector map of the completed pCd100 and pCd102 plasmids, with main regions indicated.

Figure 14: A diagrammatic summary of the construction of the pCd100 and pCd102 plasmids. Regions in colour correspond to the labelled regions in **Fig. 13**. Grey indicates the *GAL7* ORF.

4.2 Construction of transient CRISPR-Cas9 plasmids for *C. neoformans* and *C. deneoformans*

4.2.1 The pSDMA57 plasmid and removal of the Safe Haven regions

Since Fan and Lin (2018) had success with the use of a G418 resistance marker when delivering CRISPR-Cas9 plasmids into cells with electroporation, it was decided to acquire the pSDMA57 plasmid containing such a marker. The focus shifted from here on onwards to developing plasmids for *C. deneoformans* due to the low transformation efficiency of *C. neoformans* obtained with electroporation. It was also decided to abandon the hygromycin B resistance marker in favour of the G418 resistance marker. The plasmids constructed in this section were to be maintained episomally in the nucleus, without integration into the *C. deneoformans* genome. The Safe Have region was therefore removed from the *HsCAS9* plasmids constructed in the previous section to reduce the size of the plasmids, as well as from the newly obtained pSDMA57 plasmid. This was achieved by amplifying the region around the Safe Haven region and by removing the remainder with *Avr*II restriction digest reaction. Screening revealed that removal was successful (**Fig. 15**).

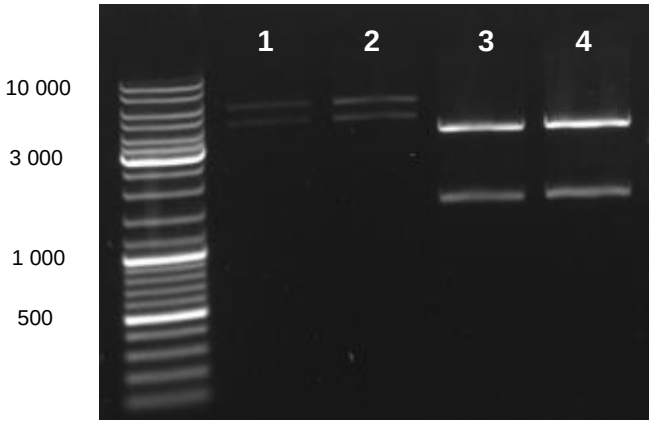

Figure 15: Lanes 1 and 2 show the pCd102 plasmid without the Safe Haven region, while lanes 3 and 4 show the pSDMA57 plasmid without this region. These plasmids were digested with *Nco*I to confirm successful removal of this region.

4.2.2 Insertion of the *HsCAS9* gene and *C. deneoformans GAL7* expression components into pSDMA57

To insert the *HsCAS9* gene into the pSDMA57 plasmid, the *HsCAS9* gene flanked by the *C. deneoformans GAL7* promoter and terminator was removed from the pCd102 plasmid by digesting with *Sac*II and *Sal*I. The *HsCAS9* fragment was then ligated into the pSDMA57 plasmid, giving the plasmid pCd104. To evaluate successful ligation, the resulting plasmid was digested with *Bgl*II and the digest products were analysed on an agarose gel, which showed a successful ligation (**Fig. 16**).

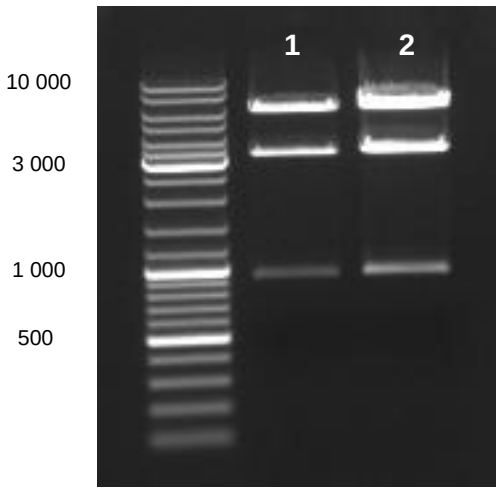

Figure 16: Lanes 1 and 2 show the pCd104 plasmid digested with *Bgl*II to show successful ligation of the *HsCAS9* and flanking regions into this plasmid.

4.2.3 Construction of pCd105 and insertion of ribozyme genes

The *C. deneoformans GAL7* gene was amplified from genomic DNA and cloned into a cloning vector. The region around the ORF was PCR amplified to obtain a fragment without the ORF. A fragment containing the hammerhead ribozyme gene, a GFP gDNA region and a hepatitis delta virus ribozyme gene was PCR amplified from the pRS316-RGR-GFP plasmid and cloned into the vector to replace the *C. deneoformans GAL7* ORF. Screening for the insert revealed that cloning was successful (**Fig. 17**). The resulting plasmid was labelled as pCd105, one of four components of the completed CRISPR-Cas9 system developed in this study. This

plasmid serves as a vector for the construction of gDNA, which is then transferred to the main CRISPR-Cas9 constructs.

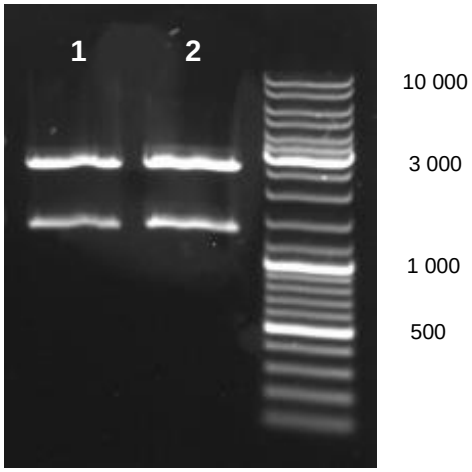

Figure 17: Lanes 1 and 2 show the hammerhead and HDV ribozymes in place of the *C. deneoformans GAL7* ORF in the pMiniT 2.0 vector. The plasmid was digested with *Eco*RI to evaluate successful cloning.

The first 20 base pairs of the gRNA molecule at the 5'-end are complementary to the target gene sequence and directs the Cas9 nuclease to its digestion site. The 3'-end of the gRNA contain sequences necessary for secondary structure formation for binding and other activities with the Cas9 nuclease (Gao & Zhao, 2014). It would, therefore, be detrimental to the function of gRNA if other unnecessary bases are found at the ends of this molecule. This poses a problem, as transcription by an mRNA producing polymerase (such as RNA polymerase II in eukaryotes) cannot be used for the synthesis of gRNAs as the product of such a polymerase undergoes extensive processing and modification at both ends of the molecule. These RNA molecules are also transported to the cytoplasm, while gRNA would be needed in the nucleus. Other promoters, such as the U3 and U6 promoters that recruit a non-mRNA producing polymerase (such as RNA polymerase II in eukaryotes), are therefore used for the synthesis of gRNA when a gDNA gene is artificially introduced into cells. These promoters are however not well characterised in all organisms, which significantly complicates the transcription of gDNA in some cells. Furthermore, the snRNA genes natively under the control of these promoters are housekeeping genes and are therefore constitutively expressed. The hammerhead and hepatitis

delta virus ribozymes were consequently used by Gao and Zhao (2014) for the proper expression and processing of gRNA and was also the approach used in this study. This allowed for the use of a *GAL7* promoter and terminator, with induced expression when cells are grown on media with galactose as a carbon source. Expression of the CRISPR-Cas9 components can therefore be controlled. These ribozymes are self-cleaving upon transcription and release a gRNA molecule with properly defined ends. The hammerhead ribozyme was attached to the 5'-end of the gDNA target site with 6 bp complementary to the first 6 bp of the target site for proper secondary structure formation required for self-cleavage. The HDV ribozyme was attached to the 3'-end of the gDNA (**Fig. 18**).

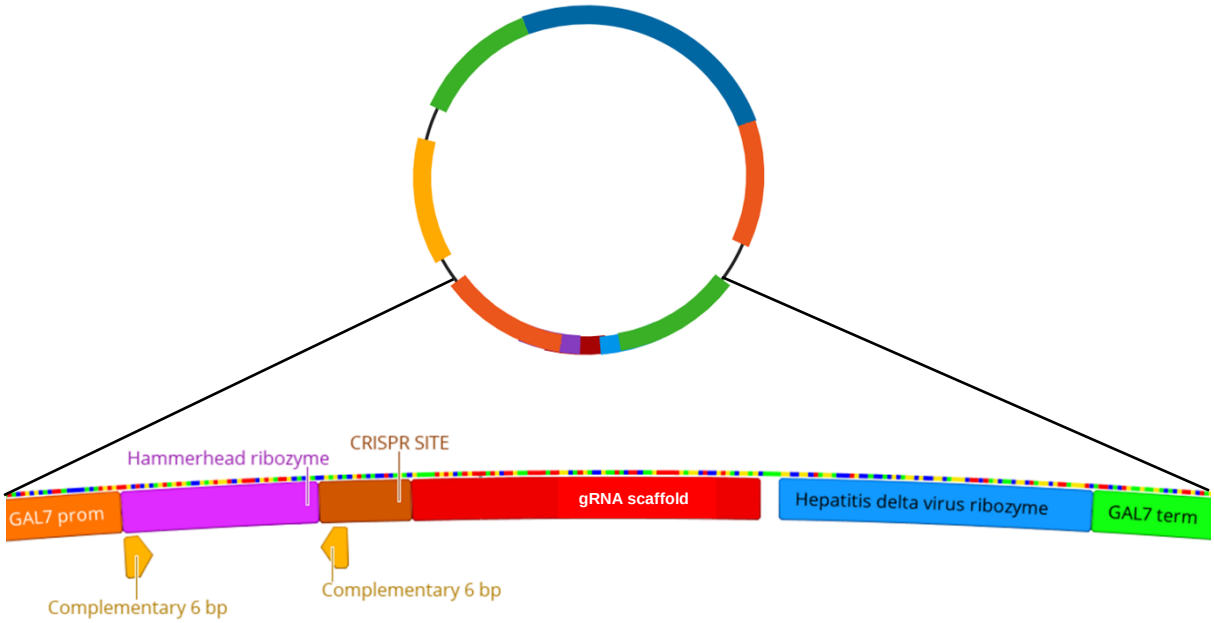

Figure 18: A section of a vector map showing the *C.deneoformans GAL7* promoter and terminator flanking the hammerhead and HDV ribozyme genes as well as the gDNA (Vector map generated using Geneious R10).

4.2.4 Construction of pCd106, pCd107 and pCd108 as well as *C. deneoformans ADE2* gDNA

To target the *ADE2* gene in *C. deneoformans*, a 20 bp region in this gene was selected with the first 6 bp being complementary to the 6 bp of the hammerhead ribozyme used to target the GFP gene, as found in the pRS316-RGR-GFP plasmid. The *ADE2* gene encodes phosphoribosylaminoimidazole carboxylase, an enzyme that catalyses a reaction in the nucleotide biosynthesis pathway. This target was selected for testing the developed CRISPR-Cas9 system as a red pigment accumulates inside *ade2*△ mutants, making the identification of *ade2*△ mutants growing on minimal media without adenine straightforward. The *ADE2* gDNA was constructed with two primers with overhangs containing the *ADE2* gDNA. These primers were used to PCR amplify the region around the GFP gDNA, following ligation to recircularise the plasmid, the newly constructed plasmid was labelled as pCd106. The promoter and terminator region flanking the ribozyme gene and *ADE2* gDNA was PCR amplified and cloned into the pCd103 and pCd104 plasmids, resulting in the pCd107 and pCd108 plasmids. Screening revealed that cloning was successful (**Fig. 19 & 22**).

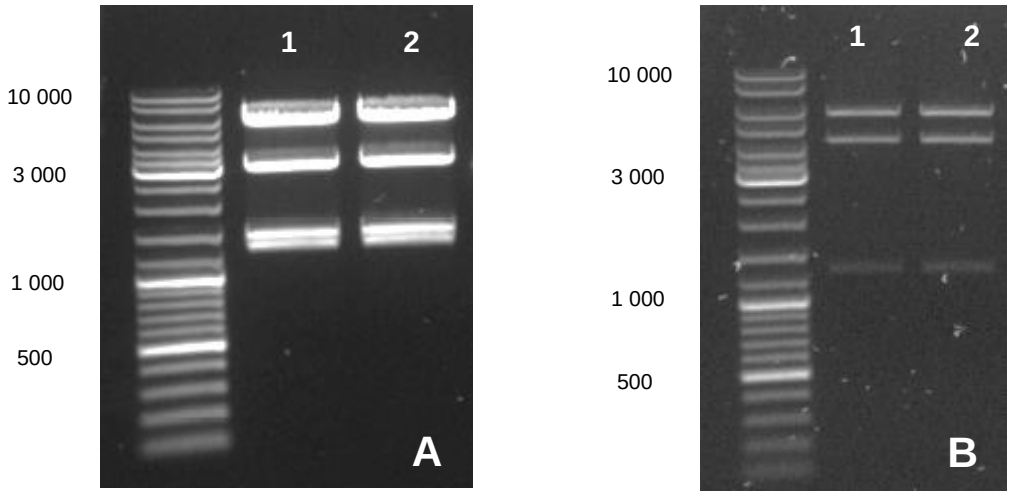

Figure 19: The pCd108 and pCd107 plasmids are shown in lanes 1 and 2 in A and B respectively. The plasmids were digested with *Nco*I to evaluate if cloning was successful.

4.2.5 Construction of pCd109, pCd110 and pCd111 as well as *C. deneoformans HIS3* gDNA

For the construction of the *C. deneoformans HIS3* gDNA, a different approach was used. Two oligonucleotides that overlap with one another were polymerised with PCR to construct the *HIS3* gDNA. For this target, a 20 bp complement was chosen with a *Pst*I restriction site to allow for screening of successful cloning. The resulting PCR polymerised oligonucleotide (**Fig. 20**) was cloned into the pCd105 plasmid to obtain the pCd109 plasmid. The *GAL7* promoter and terminator flanking the ribozyme genes as well as the *HIS3* gDNA in pCd109 was transferred to the pCd103 and pCd104 plasmids, which gave pCd110 and pCd111 respectively (**Fig. 22 and 23**). Screening for the insert revealed that cloning was successful (**Fig. 21**). The *HIS3* gene encodes imidazoleglycerol-phosphate dehydratase, which plays a role in histidine biosynthesis. Mutants lose the ability to grow on histidine-deficient media.

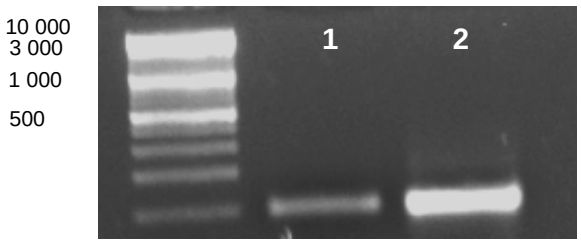

Figure 20: Lanes 1 and 2 show the PCR polymerised *C. deneoformans HIS3* gDNA oligonucleotide.

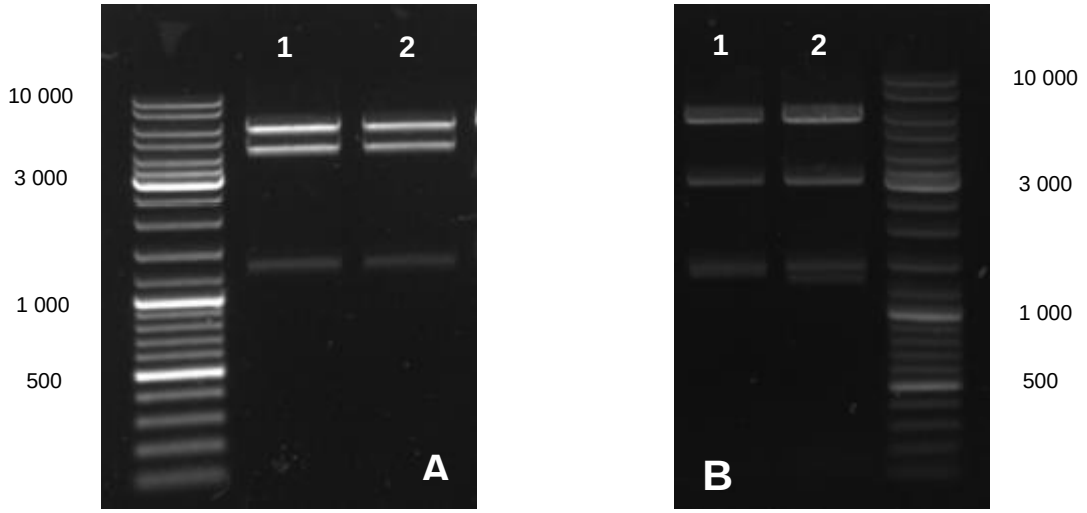

Figure 21: The pCd110 and pCd111 plasmids are shown in lanes 1 and 2 in A and B respectively. The plasmids were digested with *Nco*I to evaluate if cloning was successful.

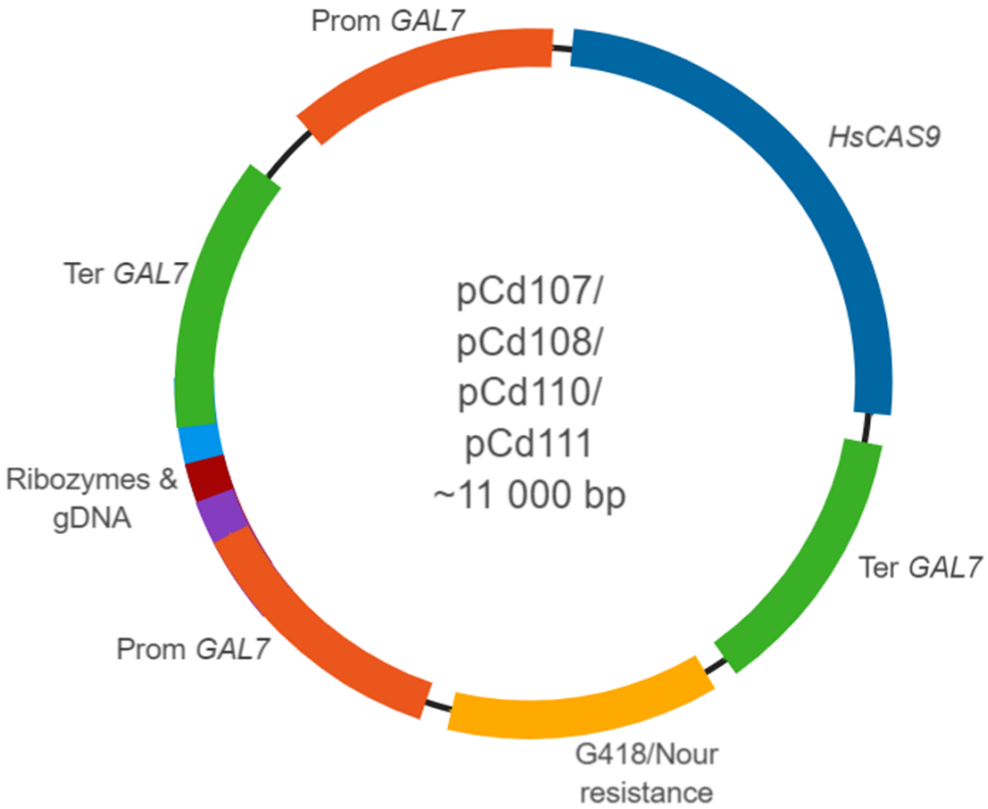

Figure 22: A simplified vector map of the completed pCd107, pCd108, pCd110 and pCd111 plasmids, with main regions indicated.

Figure 23: A diagrammatic summary of the process followed to construct the pCd107, pCd108, pCd110 and pCd111 plasmids. Regions in colour correspond to the labelled regions in **Fig. 22**.

4.3 Construction of *C. deneoformans ADE2* and *HIS3* donor DNA

Donor DNA was constructed for integration at the CRISPR-Cas9 cut sites in the *C. deneoformans ADE2* and *HIS3* genes. The *ADE2* donor DNA is 436 bp in length while the *HIS3* fragment is 308 bp in length (**Fig. 24**). Homologous recombination with these donor DNA fragments will result in the deletion of a part of each of the targeted genes and will not introduce a selectable marker into these genes.

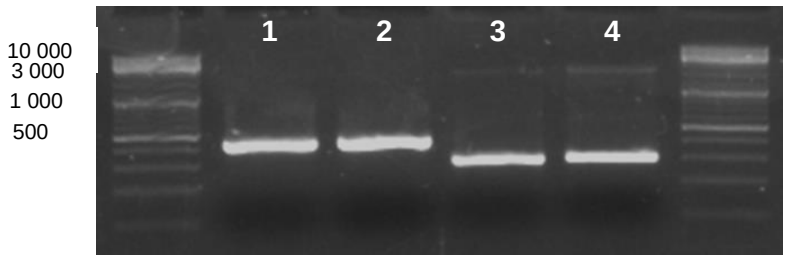

Figure 24: Lanes 1 and 2 show the PCR amplified *ADE2* donor DNA fragment, while lanes 3 and 4 show the *HIS3* donor DNA fragment.

4.4 The complete system

The complete CRISPR-Cas9 system for *C. deneoformans* consists of four components; the pCd103 and pCd104 plasmids containing the *HsCAS9* gene with a nourseothricin and G418 resistance marker respectively, the pCd105 plasmid and a suitable donor DNA fragment (**Fig. 25**). To use this system, a 20 bp CRISPR target site is selected, preferably with a restriction site to ease the screening process for plasmids with the correct insert. The complement of the target site and the gRNA scaffold is made with two overlapping oligonucleotides polymerised with PCR. The resulting fragment is then cloned into the pCd105 plasmid in between the two ribozyme genes. This region, including the flanking *GAL7* promoter and terminator, is then PCR amplified and cloned into the pCd103 and pCd104 plasmids. Donor DNA is constructed by PCR polymerising overlapping oligonucleotides. All of the plasmids constructed for *C. deneoformans* to obtain the final CRISPR-Cas9 system for this yeast are shown in **Table 3**.

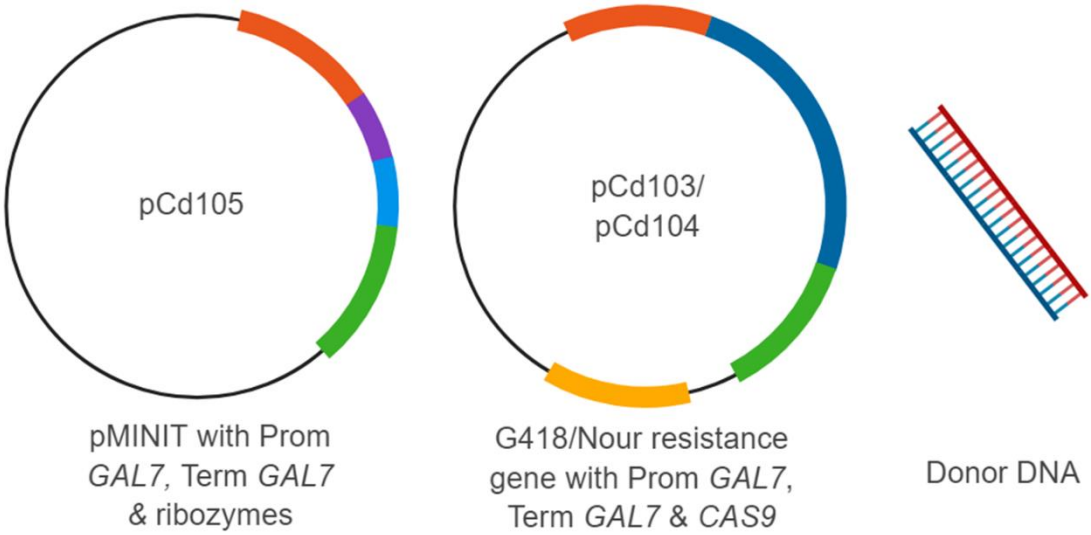

Figure 25: The four components of the CRISPR-Cas9 system developed in this study.

Table 3: All of the CRISPR-Cas9 plasmids constructed in this study.

Plasmid name	Description
pCd100	*C. deneoformans GAL7* ORF replaced with *HsCAS9* and inserted into pSDMA58
pCd102	*C. deneoformans GAL7* ORF replaced with *HsCAS9* and inserted into pSDMA25
pCd103	pCd100 with Safe Haven site removed
pCd104	*C. deneoformans GAL7* ORF replaced with *HsCAS9* and inserted into pSDMA57, Safe Haven site removed
pCd105	pMiniT 2.0 containing the *C. deneoformans GAL7*. The *GAL7* ORF replaced with the hammerhead and hepatitis delta virus ribozymes
pCd106	pCd105 with *C. deneoformans ADE2* gDNA
pCd107	pCd103 with *C. deneoformans ADE2* gDNA
pCd108	pCd104 with *C. deneoformans ADE2* gDNA
pCd109	pCd105 with *C. deneoformans HIS3* gDNA
pCd110	pCd103 with *C. deneoformans HIS3* gDNA
pCd111	pCd104 with *C. deneoformans HIS3* gDNA

5. Conclusions

Cryptococcus neoformans and *C. deneoformans* are responsible for the vast majority of cryptococcal infections worldwide (Chayakulkeeree & Perfect, 2008). Disrupting genes could help researchers to understand how these pathogens cause disease, which could, in turn, lead to new, and very much needed treatment options. One way of disrupting genes is with the CRISPR-Cas9 system. At the time when this

study was conceptualised, one such a CRISPR-Cas9 gene targeting approach for *C. deneoformans* was already developed by Wang and co-workers (2016). This system relied on non-integrative plasmids that were lost after expression – this mitigated problems with cytotoxicity, off-target digestion and effect on virulence of overexpressed *CAS9*. A later approach by Arras and co-workers (2016) relied on the integrative pSDMA plasmids which were also partly used in this study. This system was developed for *C. neoformans* and no problems with the overexpression of *CAS9* were found when this gene is integrated into the genome. A similar approach to the one employed by Arras and co-workers (2016) was used in this study, but was applied to *C. deneoformans* instead. Integrative plasmids were also developed for *C. neoformans* for comparison. Transiently maintained plasmids containing all of the CRISPR-Cas9 components on a single plasmid were also developed for *C. deneoformans*. This latter approach includes two plasmids with two distinct antibiotic resistance markers to allow for a second round of transformation in order to restore function to the targeted gene. This allows for the fulfilment of Falkow's molecular Koch's postulates (Falkow, 1988).

6. References

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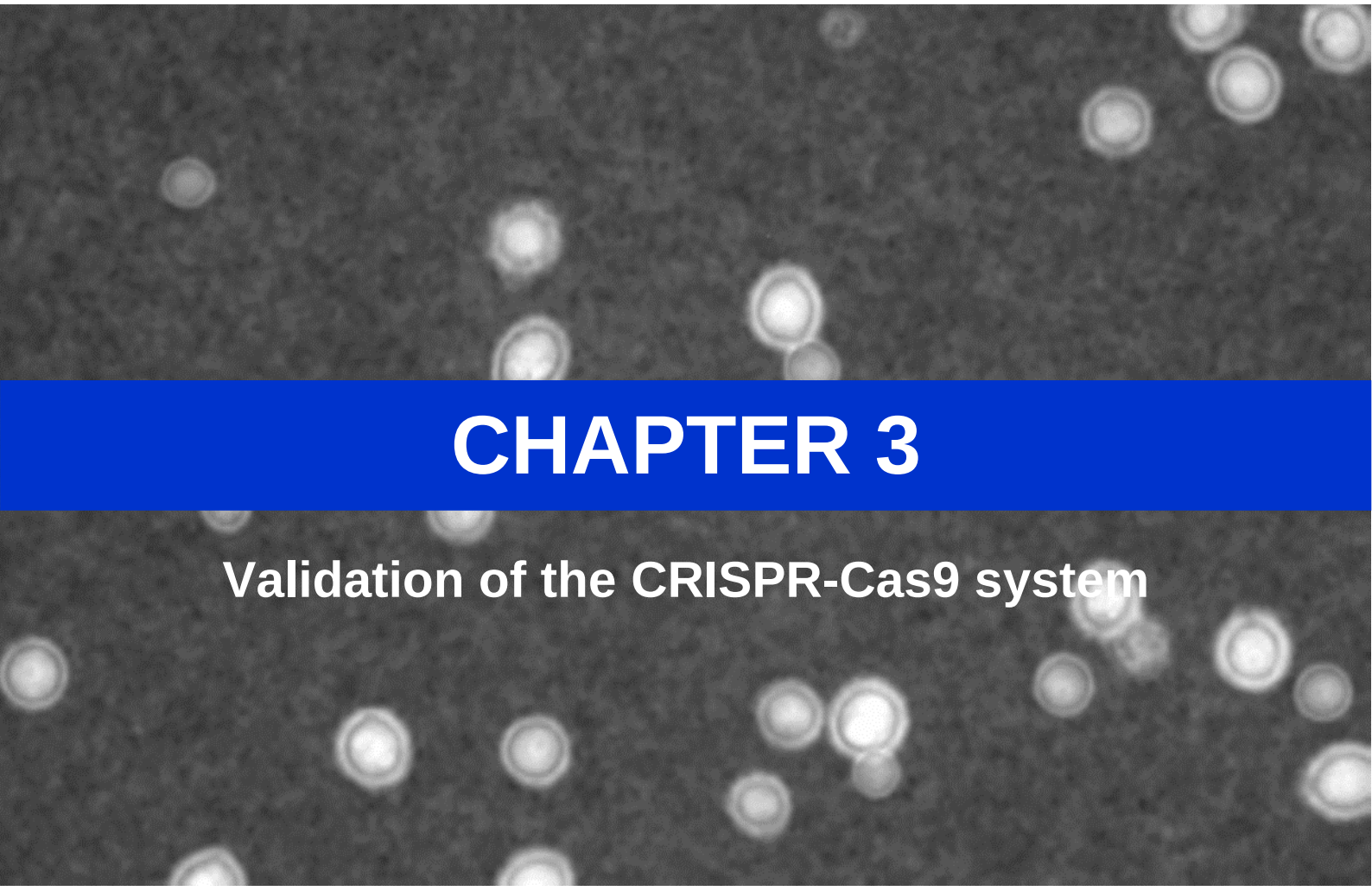

CHAPTER 3

Validation of the CRISPR-Cas9 system

1. Abstract

Since *Cryptococcus neoformans* and its sibling, *C. deneoformans*, are covered with a thick capsule, only physical transformation methods seem to be effective in delivering DNA into the cells. While biolistic transformation is effective in both species, electroporation seems to be more effective for *C. deneoformans* than *C. neoformans*. In this study, *C. neoformans* could not be successfully transformed with an integrative *CAS9* plasmid. Limited success was obtained with an integrative *CAS9* plasmid for *C. deneoformans* when delivered with electroporation, although stability tests showed that the plasmid was not integrated into the genome and the transformants did not remain stable. Electroporation of *C. deneoformans* cells to deliver non-integrative CRISPR-Cas9 plasmids yielded very few colonies and none of the colonies remained stable. The expression and functioning of the CRISPR-Cas9 components could also not be demonstrated and another approach should be implemented in the future.

2. Introduction

Delivering plasmids into cells for gene targeting relies on an efficient transformation protocol. Since the yeast *Saccharomyces cerevisiae* is a well-established industrial and scientific workhorse, developing transformation protocols for this organism seemed logical. Oppenoorth (1960) was the first to attempt transformation on *S. cerevisiae*, although many others could not replicate his findings (Harris & Thompson, 1960). The first effective protocol was developed by Hinnen and co-workers (1978) who enzymatically treated *leu2*△ mutant cells to make spheroplasts. The spheroplasts were stabilised with sorbitol and then mixed with polyethylene glycol (PEG) and a plasmid carrying a *LEU2* gene to restore prototrophy in transformed mutants. While the above plasmid had to be integrated into the genome to be expressed, Beggs (1978) developed an autonomously replicating plasmid for expression in this yeast by including an endogenous autonomously replicating yeast 2 µm circle into a bacterial plasmid. This plasmid was delivered into the cells with the same spheroplast protocol developed by Hinnen and co-workers (1978).

Although the spheroplast method soon became the gold standard for yeast transformation, it suffered from drawbacks such as the need for regeneration agar (Gietz & Woods, 2001). This made replica plating difficult and the development of other intact cell transformation protocols soon became a better alternative. Iimura and co-workers (1983) reported that treating *S. cerevisiae* cells with calcium chloride ($CaCl_2$) induced the cells to take up plasmids. Ito and co-workers (1983) showed that specific cations, such as Na^+ (sodium), K^+ (potassium), Rb^+ (rubidium), Cs^+ (caesium), and Li^+ (lithium) could be used in combination with PEG to stimulate plasmid uptake by yeast cells. They found that PEG, which is thought to be responsible for the absorption of DNA to the yeast cell wall, and a heat shock step at 42°C were essential. Lithium acetate as a source of cations was shown to be the most effective. This protocol was continuously refined over the following decade, with concentrations optimised and the addition of other substances, such as carrier DNA to enhance efficacy (Gietz & Woods, 2001).

Physical transformation methods such as electroporation and biolistic transformation were also employed to deliver DNA into yeast cells. Electroporation was first used to deliver DNA into mouse cells by Neumann and co-workers (1982) and was later also used for plant and bacterial cells (Calvin & Hanawalt, 1988; Bates, 1995). This transformation method involves exposing cells to an electrical impulse which then presumably result in the formation of pores in the cell wall and membrane to allow entry to macromolecules, such as DNA (Neumann *et al*., 1982). Another approach to yeast transformation is to agitate cells, plasmid and carrier DNA with glass beats (Costanzo & Fox, 1988). This method badly damages the cells and transformants are only obtained when cells are plated in the presence of sorbitol, an osmotic protector, on selective media. This protocol is not frequently employed due to its inefficiency. Biolistic transformation is an alternative method that involves using gas under pressure to shoot metal microparticles into cells (Gietz & Woods, 2001). This was first used by Klein and co-workers (1987) to transform plant cells and made its way to yeast cells in 1988 when Johnston and co-workers (1988) used it to deliver DNA into the mitochondria of *S. cerevisiae*.

Since the pathogenic yeast *Cryptococcus neoformans* and its pathogenic siblings are covered by a thick capsule, physical transformation methods are almost exclusively used to deliver DNA into these cells. In this chapter, electroporation is employed to deliver the CRISPR-Cas9 system described in the previous chapter into cryptococcal cells to test the developed gene targeting system.

3. Materials and methods

3.1 PCR amplification and primers used

Polymerase chain reaction (PCR) amplification of genes and fragments was a routine procedure during this study. The specific conditions are given where applicable and the names and sequences of the primers used throughout this chapter are given in **Table 4**.

Table 4: Primers used in chapter 3.

Primer name	Primer sequence
Cn-25srRNA-1F	AAGTACCGTGAGGGAAAGATGA
Cn-25srRNA-1R	TTGGCTGTGGTTTCGCTAGATA
Safe haven-1F	ATGTTTTCCATCAGTAACATCGGGG
Safe haven-1R	CTGTGCCAAGTGGATTGCTTATGC
HsCas9-1F	TGGAGGAGTCCTTTTTGGTG
HsCas9-1R	AAAATTCCTCCTGGCTTGCT

3.2 Determination of the minimum inhibitory concentration (MIC) of antibiotics used for selection

A loopful of *C. neoformans* and *C. deneoformans* cells were used to inoculate two separate tubes with 5 mL YPD broth (10 $g.L^{-1}$ yeast extract, 20 $g.L^{-1}$ peptone, 20 $g.L^{-1}$ D-glucose). The cells were incubated overnight at 30°C while shaking. This culture (200 µL) was spread on YPD agar plates containing either hygromycin B (Merck Millipore) or nourseothricin (Jena Bioscience) in the following series of concentrations: 25 $mg.L^{-1}$, 50 $mg.L^{-1}$, 75 $mg.L^{-1}$, 100 $mg.L^{-1}$, 125 $mg.L^{-1}$, 150 $mg.L^{-1}$ and 200 $mg.L^{-1}$. Later on in the study, another MIC test was done to test the growth *of C. deneoformans* in the presence of geneticin (G418, Sigma-Aldrich). The following series of concentrations were used: 10 $mg.L^{-1}$, 25 $mg.L^{-1}$, 50 $mg.L^{-1}$, 75 $mg.L^{-1}$, 100 $mg.L^{-1}$, 125 $mg.L^{-1}$, 150 $mg.L^{-1}$, 175 $mg.L^{-1}$ and 200 $mg.L^{-1}$.

3.3 Linearisation of constructed plasmids for transformation

The *HsCAS9* plasmids constructed for *C. neoformans* and *C. deneoformans* were linearised by PCR amplifying the circular plasmids prior to transformation via electroporation into these yeast cells. For the *C. deneoformans HsCAS9* constructs with the Safe Haven region, this was achieved by using a KOD Hot Start Polymerase kit (Novagen) and the following parameters: an initial denaturation step at 94°C maintained for 2 min; a denaturation step at 95°C kept for 20 sec, an annealing step at 52°C kept for 10 sec and an elongation step at 70°C kept for 150 sec repeated for 25 cycles and a final elongation step at 72°C kept for 7 min. The primer pair Safe haven-1F (forward) and Safe haven-1R (reverse) was used for amplification. The *C. neoformans HsCAS9* constructs with the Safe Haven region were amplified for

linearisation by using the same KOD Hot Start Polymerase kit (Novagen) and program as well as the same primer set. For linearisation of the *C. deneoformans HsCAS9* constructs with the 25S rRNA region, the KOD Hot Start Polymerase kit (Novagen) was used with an annealing temperature of 50°C maintained for 10 sec. The primer pair Cn-25srRNA-1F (forward) and Cn-25srRNA-1R (reverse) was used. The resulting fragments were separated on a 0.8% (w/v) agarose gel, excised and purified using a Qiagen QIAquick Gel Extraction kit. The extracted plasmids were resuspended in deionised H_2O, instead of elution buffer provided by the kit, to minimise salt in the suspension. A NanoDrop™ microvolume spectrophotometer (Thermo Fisher Scientific) was used to determine the concentration of the linearised plasmids before transformation.

To linearise the constructs containing both the *HsCAS9* gene as well as the *C. deneoformans* gDNA sequences (plasmids pCd107, pCd108, pCd110 and pCd111), restriction digest was done with *Xho*I (Thermo Fisher Scientific). All four of the linearised fragments were analysed on a 0.8% (w/v) agarose gel, excised and purified using a GeneJET Gel Extraction kit (Thermo Fisher Scientific). After purification, plasmids were resuspended in deionised H_2O to minimise salt in the suspension. A NanoDrop™ microvolume spectrophotometer (Thermo Fisher Scientific) was used to determine the concentration of the linearised plasmids.

3.4 Transforming *C. deneoformans*

3.4.1 Transforming with an electroporation method optimised for *Cryptococcus* spp.

A protocol developed by Wickes and Edman (1994) for the electroporation of cryptococcal cells was followed in this study. A loopful of cryptococcal cells (*C. neoformans* or *C. deneoformans*) growing on a YPD plate was used to inoculate 30 mL YPD or YPGalactose (containing 20 $g.L^{-1}$ galactose instead of D-glucose) broth in a conical flask. Overnight incubation at 30°C in an orbital shaker followed. The overnight culture (2 mL) was used to inoculate 200 mL YPD or YPGalactose broth in a conical flask, which was incubated at 30°C while shaking in an orbital shaker. The

optical density (OD) was measured with a spectrophotometer (BioPhotometer, Eppendorf) at a wavelength of 600 nm until an OD_{600} of about 1 was reached. This culture (100 mL) was collected in a conical centrifuge tube by centrifugation at 3000 x *g* for 5 min while maintaining a temperature of 4°C. The cells were washed twice with ice-cold distilled H_2O, centrifuging as before. Pelleted cells were then resuspended in 50 mL cooled electroporation buffer containing 10 mM Tris-HCl at pH 7.5; 1 mM $MgCl_2$ and 270 mM Sucrose. Freshly prepared 1 M Dithiothreitol (DTT) (200 µL) was added and the cells were incubated on ice for 30 min. Cells were washed in 50 mL electroporation buffer without the addition of DTT and centrifuged. The supernatant was discarded leaving only approximately 1 mL of electroporation buffer for resuspension of the cells. The resuspended cells were divided into three 1,5 mL microfuge tubes so that each tube contain about $3x10^8$ cells. Cells were centrifuged at 3000 x *g* for 5 min at 4°C and the supernatant was decanted leaving about 40 µL for resuspension. Linearised plasmid DNA (and donor DNA when applicable) were added to the resuspended cells in different amounts, ranging from 250 ng to 3200 ng. This mixture was transferred to a 0.2 cm gap sterile electroporation cuvette and placed in the shocking chamber of a Bio-Rad Gene Pulser® 1652076 electroporator coupled to a pulse controller. The following parameters were set: voltage at 0.5 kV or 2 kV; capacitance at 25 µF and resistance at 200 Ω or 1000 Ω. An electrical pulse was sent through the cells and the time constant was recorded. YPD or YPGalactose broth (1 mL) was added to electroporated cells in the cuvette before transferring to a 1.5 mL microfuge tube. Cells were incubated at 30°C for 3 hours while shaking. Cells were pelleted by centrifugation to remove excess medium and the entire cell suspension was plated onto YPD or YPGalactose plates containing either 100 $mg.L^{-1}$ nourseothricin, 100 $mg.L^{-1}$ hygromycin B or 200 $mg.L^{-1}$ G418. Plates were incubated at 30°C for 3 to 4 days. This was done in duplicate for each plasmid. Colonies growing on these plates were transferred to minimal medium agar plates prepared with yeast nitrogen base (YNB) without adenine or histidine.

3.4.2 Transforming with an electroporation method optimised for *Komagataella phaffii* (*Pichia pastoris*)

Another approach to electroporation was to follow a method developed by Wu and Letchworth (2004) for the yeast *Komagataella phaffii* (previously known as *Pichia pastoris*). In this protocol, electroporation is combined with chemically treating the cells to enhance transformation efficiency. A loopful of *C. deneoformans* cells growing on YPD agar was used to inoculate 3 mL YPD broth in a test tube. The tubes were incubated at 30°C overnight while shaking. The overnight culture (1 mL) was transferred to 100 mL YPD broth in a 250 mL conical flask. This fresh culture was incubated at 30°C on an orbital shaker until an OD_{600} of between 1 and 2 was reached. Approximately $8x10^8$ cells were harvested, since an OD_{600} of 1 is about $5x10^7$ cells.mL^{-1}, 11 mL of the above culture was transferred to a 15 mL conical centrifuge tube. Cells were pelleted by centrifugation at 3000 x *g* for 5 min and the supernatant was decanted. Cells were then washed with 8 mL of distilled H_2O, centrifuging as before. The cells were resuspended in 8 mL electroporation buffer containing 0.1 M lithium acetate; 0.6 M Sorbitol; 0.01 M Tris-HCl at pH 7.5. Freshly prepared DTT (80 µL of a 1 M stock) was added and the cells were incubated at room temperature for 45 min. The cells were harvested as before and were resuspended in 1.5 mL 1 M ice-cold sorbitol and transferred to a 2 mL microfuge tube. To discard the sorbitol, the cells were centrifuged as before at 4°C. Washing with sorbitol was done twice. Cells were pelleted and resuspended in 50 µL ice-cold sorbitol, which should give a concentration of $1x10^{10}$ cells.mL^{-1}. Circular plasmid DNA (300 ng) and 600 ng donor DNA was added to the cells suspended in sorbitol. The mixture was incubated on ice for 5 min before being transferred to a sterile 0.2 cm gap cuvette. The cuvette was placed in the shocking chamber of a Bio-Rad Gene Pulser® 1652076 electroporator coupled to a pulse controller. The following parameters were set: voltage at 1.5 kV; capacitance at 25 µF and resistance at 200 Ω. An electrical pulse was sent through the cells and the time constant was recorded. Sorbitol (500 µL of a 1 M stock) and 500 µL YPD or YPGalactose broth were added to the cells in the cuvette. The cell suspension was transferred to a 1.5 mL microfuge cuvette and incubated at 30°C for 3 hours while shaking. Excess medium was removed by centrifuging the cells and the entire cell suspension was plated onto YPD or YPGalactose plates containing either 100 $mg.L^{-1}$ nourseothricin

or 200 mg.L^{-1} G418. Plates were incubated at 30°C for 3 to 4 days. This was done in duplicate for each plasmid. Colonies growing on these plates were transferred to minimal medium agar plates.

3.4.3 Transforming *C. deneoformans* with a lithium acetate-based method

A chemical-based transformation protocol often used for yeast was also employed to transform *C. deneoformans*. This protocol was described by Nguyen and co-workers (2017) to transform *Candida albicans*. A loopful of cells growing on a YPD agar plate was used to inoculate 5 mL YPD broth in a test tube and incubated at 30°C overnight while shaking. The overnight culture (100 µL) was used to inoculate fresh 5 mL YPD broth in a test tube, which was incubated at 30°C while shaking until the cell density reached an OD_{600} of 0.5 to 0.8. Cells from two test tubes were harvested in a 15 mL conical centrifuge tube by centrifuging at 3400 x *g* for 5 min. The cells were resuspended in 1 mL distilled H_2O. The suspension was transferred to a 2 mL microfuge tube and centrifuged at 3400 x *g* for 1 min. The cell pellet was resuspended in 100 µL distilled H_2O and 50 µL of the cell suspension, 10 µL carrier DNA (10 mg.mL^{-1} denatured salmon sperm DNA), 2000 ng linearised plasmid DNA and 44 µL unpurified donor DNA (PCR product) were mixed together. Freshly prepared transformation mix (1 mL; 875 µL 50% (w/v) PEG 3350, 100 µL 10x TE buffer [100 mM Tris at pH 7.4 and 10 mM EDTA at pH 8] and 25 µL 1 M lithium acetate at pH 7) were added and the mixture was gently mixed by inverting the tubes. The cell suspension was incubated overnight at 30°C while shaking. The cells were then heat shocked at 45°C for 15 min and centrifuged at 4000 x *g* for 2 min. The supernatant was aspirated completely, and cells were washed with 1 mL YPGalactose broth. Cells were centrifuged as before, and the cell pellet was resuspended in 2 mL YPGalactose broth. Following resuspension, cells were incubated at 30°C for 4 hours while shaking. The cells were centrifuged to remove excess media and the entire cell suspension was plated on YPGalactose plates containing either 100 mg.L^{-1} nourseothricin or 200 mg.L^{-1} G418, depending on the resistance gene on the plasmid. Plates were incubated at 30°C for 3 to 4 days. This was done in duplicate for each plasmid. Colonies growing on these plates were transferred to minimal medium agar plates.

3.5 Validating transformation of *C. deneoformans*

3.5.1 Colony PCR to validate the presence of *HsCAS9* gene in *C. deneoformans*

To test if the *HsCAS9* gene was successfully delivered into *C. deneoformans* cells, colony PCR was performed. A pipette tip was used to pick up a colony of cells growing on a YPD + 100 $mg.L^{-1}$ hygromycin B agar plate after transformation. The cells were suspended in 30 µL distilled PCR-grade H_2O and mixed with a vortex mixer for 20 sec. The cells were heated at 90°C for 5 min, followed by more mixing for 10 sec. PCR reagents from a KAPA Taq PCR kit were added following the provided instructions. The following program was followed: initial denaturation at 95°C for 3 min, denaturation at 95°C for 30 sec, annealing at 50°C for 30 sec, elongation at 72°C for 1 min and a final elongation step at 72°C for 1 min. The denaturation, annealing and elongation steps were repeated for 35 cycles. The primer set HsCas9-1F (forward) and HsCas9-1R (reverse) was used.

3.5.2 Western blot analysis to validate the expression of *HsCAS9* gene in *C. deneoformans*

Western blot analysis was performed to test expression of the *HsCAS9* gene as well as proper functioning of the inducible *C. deneoformans GAL7* promoter. A loopful of cells growing on a YPD + 100 $mg.L^{-1}$ hygromycin B agar plate obtained after transformation was used to inoculate 5 mL YPD broth containing 200 $mg.L^{-1}$ hygromycin B, 5 mL YPD/Galactose broth (5 $g.L^{-1}$ D-glucose and 15 $g.L^{-1}$ galactose) containing 200 $mg.L^{-1}$ hygromycin B and 5 mL YPGalactose broth containing 200 $mg.L^{-1}$ hygromycin B. Untransformed *C. deneoformans* cells were also used to inoculate such a set of tubes as control. The tubes were incubated at 30°C overnight while shaking. Culture (1 mL) from each tube was transferred to tubes suitable for a bead beater. The cells were pelleted and the supernatant was discarded. Buffer (500 µL; 50 mM Tris, 0.1% (w/v) SDS and 50 mM EDTA) and a volume of 200 µL glass beads were added to the pelleted cells. The cells were processed in a bead beater (BeadBug™) for 4 min; 1 min at a time with intermitted incubation steps on ice for 1 min. Cell debris mixture 20 µL was added to 20 µL Laemmli sample buffer mixed

with β-mercaptoethanol (50 µL βME to 950 µL sample buffer). The cells were boiled at 96°C for 10 min and lightly centrifuged to remove large cell fragments. This solution (20 µL) was loaded onto an SDS-PAGE (sodium dodecyl sulphate – polyacrylamide gel electrophoresis) gel that was prepared according to manufacturer's protocol (TGX™ FastCast™ Acrylamide Kit, 10% (w/v) from Bio-Rad). The gel was run at 100 V for approximately 1.5 hours and was stained according to the Fairbanks Coomassie Blue protein staining and method (Fairbanks *et al*., 1971). The gel was imaged in a Gel Documentation™ XR+ (Bio-Rad) to evaluate the success of protein extraction.

A second, unstained, SDS-PAGE gel was prepared for western blotting analysis as described before. The gel, as well as the polyvinylidene difluoride (PVDF) transfer membrane and filter paper, were equilibrated in Towbin transfer buffer (25 mM Tris; 192 mM glycine at pH 8.3 and 20% (v/v) methanol) for approximately 10 min on a platform shaker (Rocker 35 EZ Lab Shaker). The transfer sandwich was packed with the membrane and gel in contact with each other and was analysed for 30 min at 25 V and 1 A. The transfer membrane was transferred to wash buffer containing 100 mL PBS with 0.05% (v/v) Tween-20 and incubated for 3 min. The membrane was then transferred to blocking buffer (10 g milk powder and 200 mL PBS with 0.05% (v/v) Twen-20) and placed on the platform shaker for 1 hour. Anti-CRISPR-Cas9 antibody (1 µL; Abcam) was then added to 5 mL fresh blocking buffer into which the membrane was transferred. The membrane and solution were once again placed on the platform shaker for 1 hour. The membrane was then washed in 15 mL wash buffer by placing the membrane on the platform shaker for 5 min, repeated for 6 times. The wash buffer was discarded and 3.5 mL Luminol enhancer solution (Clarity Western ECI substrate, Bio-Rad) and 3.5 mL peroxide solution (Clarity Western ECI substrate, Bio-Rad) was added to the membrane, which was placed on the platform shaker for 5 min. The membrane was removed from the solution and covered with plastic wrap. Visualisation was achieved by viewing with a ChemiDocumentation™ XRS+ (Bio-Rad).

3.5.3 Testing the stability of transformed *C. deneoformans* cells

A colony growing on a YPD + 100 mg.L^{-1} hygromycin B agar plate after transformation was used to inoculate a test tube with 5 mL YPD without hygromycin B. The tube was incubated overnight at 30°C while shaking. An inoculum from the overnight culture was used to make a 1:10 serial dilution and 100 µL from each of the tubes in the serial dilution was spread onto YPD agar plates and incubated at 30°C until single colonies could be seen. Plates with clearly distinguishable single colonies were used for replica plating onto YPD + 100 mg.L^{-1} hygromycin B plates. These plates were also incubated at 30°C until single colonies were seen. This process was repeated for a few days to test if transformants remain stable. Colonies growing on the original YPD + 100 mg.L^{-1} hygromycin B plate plated after transformation were also transferred to fresh YPD + 100 mg.L^{-1} hygromycin B plates to test maintained survival in the presence of hygromycin B.

4. Results and discussion

4.1 Transforming with *HsCAS9* plasmids for integration into the genome

4.1.1 Transformation of *C. neoformans* and *C. deneoformans* with *HsCAS9* plasmids via electroporation

An initial approach to CRISPR-Cas9 gene targeting in *C. deneoformans* and *C. neoformans* was to deliver a plasmid carrying a *HsCAS9* gene into the cells. Since only physical transformation methods can effectively transform these yeasts, electroporation was used for integration of the plasmid into the Safe Haven region of *C. neoformans* and the 25S rRNA gene of *C. deneoformans*. Purified and linearised plasmids (600 ng) were electroporated into the cells according to section 3.4.1. The time constant obtained after electroporation varied from 13 to 23 msec with values of between 15 and 25 regarded as ideal. Lower values would mean that the electrical pulse passes through the sample too quickly and could be a sign of a high salt concentration in the sample, which lowers the resistance. The time constant (τ) is the product of the resistance and capacitance in the circuit and measures for how long the sample is exposed to the electrical current.

No colonies were seen growing on the YPD + 100 mg.L^{-1} hygromycin B and YPD + 100 mg.L^{-1} nourseothricin selective agar plates after 5 days. These antibiotic concentrations were most often used in literature for the selection of cryptococcal transformants and were therefore also used in this study. The MIC studies revealed that these yeasts are very sensitive to these antibiotics and colonies were only seen on the 10 mg.L^{-1} G418 plates after incubation for 3 to 4 days. Transformation via electroporation was repeated with 500 ng of the same plasmids and the time constants once again varied from 13 to 23 msec. A positive control plate was included without antibiotics. Electroporated cells without plasmid were plated onto this plate to assess damage to cells due to electroporation. Colonies were only seen growing on the positive control plate after 5 days, indicating that the cells are not damaged by electroporation to a large extent. The lack of favourable results might be explained by the fact that homologous recombination (HR) occur at extremely low frequencies in electroporated cells - in the range of 0.00001 to 0.001% of cells - and most plasmids are therefore maintained episomally (Lin *et al*., 2014). According to Arras and co-workers (2016), *C. neoformans* is unable to maintain plasmids episomally and the plasmids are lost after only a few generations. Lin and co-workers (2014) have had some success with this yeast using a G418 antibiotic resistance marker and obtained 140 stable transformants out of a total of 164 transformants, but only two transformants underwent homologous replacement. A follow-up study with CRISPR-Cas9 plasmids delivered into *C. neoformans* with electroporation revealed that genes could be targeted with transient plasmids providing that the CRISPR-Cas9 genes are expressed before the plasmids are lost (Fan & Lin, 2018). Wang and co-workers (2016) did however find that transient plasmids are maintained for much longer in *C. deneoformans*.

With this in mind, the second approach was, therefore, to see if transient plasmids could be maintained in *C. deneoformans* after electroporation. An amount of 500 ng linearised and purified plasmid containing the *HsCAS9* gene, the *C. deneoformans GAL7* promoter and terminator, as well as with the Safe Haven region (pCd100 and pCd102) were transformed into *C. deneoformans* cells with electroporation. Time constants of 14 to 22 msec were obtained during the delivery of the electrical pulse. 5 to 6 colonies were seen on the YPD + 100 mg.L^{-1}

hygromycin B plates after 5 days and the stability of these transformants was assessed.

4.1.2 Assessing the stability of transformants and *HsCAS9* expression

To screen for the presence of the HsCAS9 gene in the transformants obtained on the YPD + 100 mg.L^{-1} hygromycin B plates, a colony PCR was set up to test for the presence of the *HsCAS9* gene in the cells. The plasmid carrying the *HsCAS9* gene was included in the PCR setup as control. Bands comparable with the band obtained from the *HsCAS9* plasmid were seen when the PCR amplified fragments were analysed on a gel (**Fig. 26**).

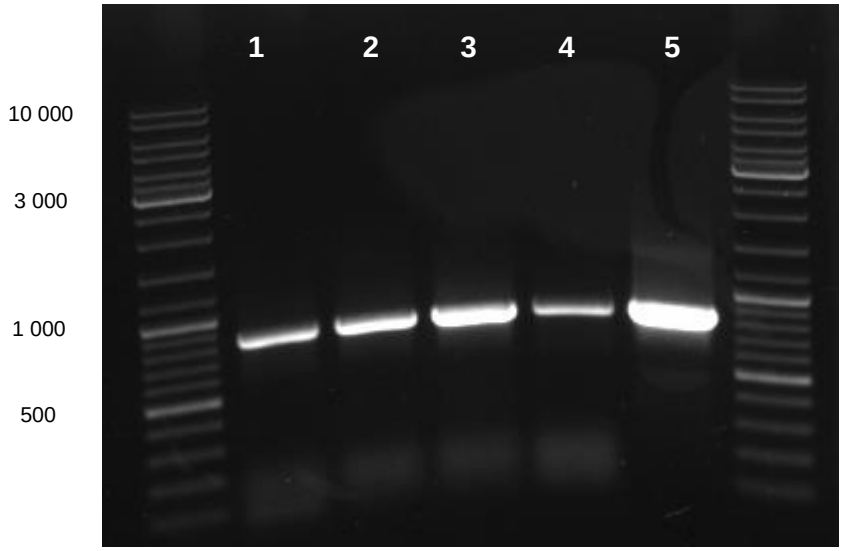

Figure 26: Lanes 1 to 4 show part of the *HsCAS9* gene amplified to screen for its presence in *C. deneoformans* transformants. For comparison, the same part was amplified from the plasmid used to transform this yeast (lane 5).

To test if the *HsCAS9* gene was expressed in *C. deneoformans* transformants, a western blot analysis was done. Cells were cultivated in YPD, YPD + galactose and YPGalactose broth overnight and total protein was extracted. The inclusion of galactose served to induce the *GAL7* promoter. Western blotting was preceded by SDS-PAGE to evaluate the success of protein extraction from the transformants. The gel showed that total protein extraction was indeed successful (**Fig. 27**). Western

blot analysis could, however, not detect any *HsCAS9* expression, neither in cells cultivated in YPD nor in YPGalactose broth (**Fig. 28**).

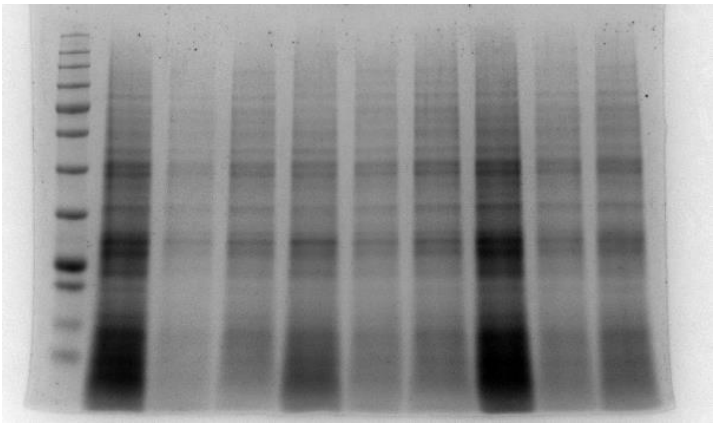

Figure 27: SDS-PAGE showed successful protein extraction from *C. deneoformans* transformants.

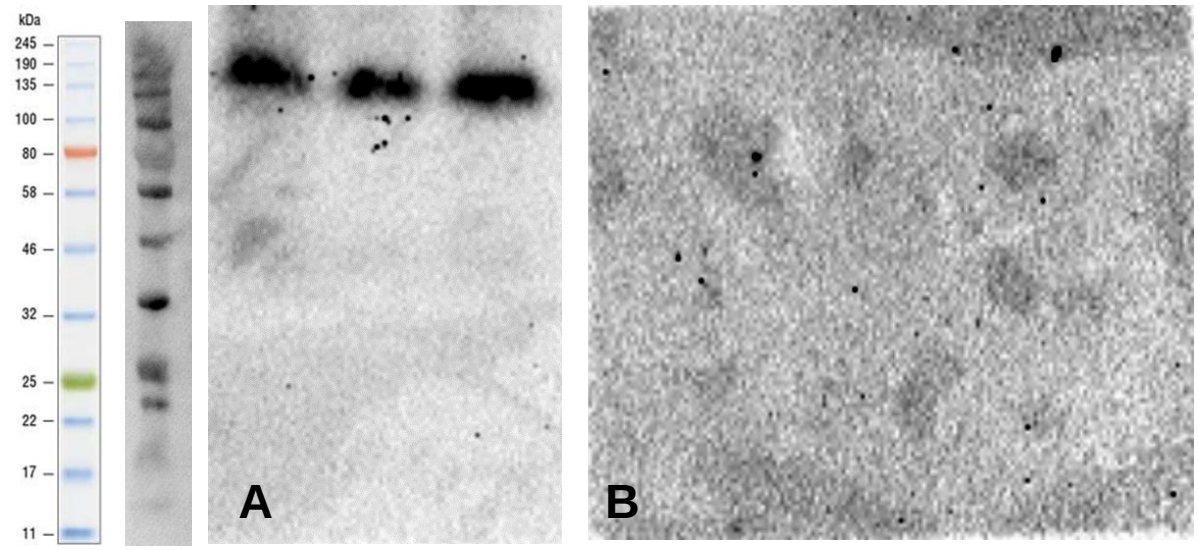

Figure 28: Western blot analysis showed no expression of the *HsCAS9* gene (B). The *CAS9* gene expressed in *Saccharomyces cerevisiae* (with a size of 160 kDa) is shown as control (A). A Colour Pre-Stained Protein Standard (from NEB®, 11 to 245 kDa) is shown on the left.

To test the stability of *C. deneoformans* transformants, the cells were passaged on YPD agar and replica plated to YPD + 100 $mg.L^{-1}$ hygromycin B plates. Colonies could be seen on the YPD plates, but no colonies formed on the plates containing hygromycin B. Transferring transformed colonies from the original YPD + 100 $mg.L^{-1}$

hygromycin B plate to a fresh YPD + 100 mg.L^{-1} hygromycin B plate showed two colonies with significant growth (**Fig. 29**). Repeating colony PCR on the cells with significant growth on the fresh plate to test for the presence of the *HsCAS9* gene revealed that the cells lost the plasmid with the *HsCAS9* gene (**Fig. 30**).

Figure 29: Colonies growing on the original YPD + 100 mg.L^{-1} hygromycin B plate were transferred to a fresh plate. Only two colonies (at the bottom, labelled 12 and 13) showed significant growth on the fresh plate.

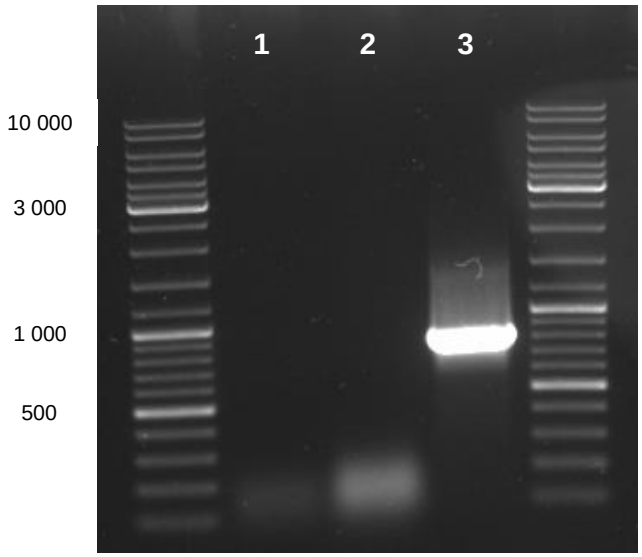

Figure 30: Lanes 1 and 2 show the absence of the *HsCAS9* gene PCR amplified from the two colonies growing on the fresh YPD + 100 mg.L^{-1} hygromycin B plate. Lane 3 show the same gene amplified from the plasmid used for transformation.

4.2 Transforming *C. deneoformans* with transiently maintained plasmids

4.2.1 Transforming via electroporation

After observing that transformants do not integrate the *HsCAS9* gene into the intended regions in the genome, transient plasmids were constructed. The rationale was that, if all of the components are transcribed and/or expressed before the plasmids are lost, the system should successfully cleave the targeted genes. Both the *HsCAS9* gene and gDNA gene were included on a single plasmid with a separate donor DNA fragment, as discussed in Chapter 2. An amount of 250 ng constructed plasmid (pCd107, pCd108, pCd110 or pCd111) together with 250 ng of the relevant donor DNA were delivered simultaneously into *C. deneoformans* cells with electroporation as described in section 3.4.1. Time constants of 13 to 15 msec were obtained, which were in the lower portion of the acceptable range of 15 to 25 msec. Cells were incubated in YPGalactose broth after electroporation for 3 hours and were plated onto YPGalatose plates with 100 $mg.L^{-1}$ nourseothricin or 200 $mg.L^{-1}$ G418 in duplicate. Only a single colony on a G418 plate was seen after 5 days (**Fig. 31**).

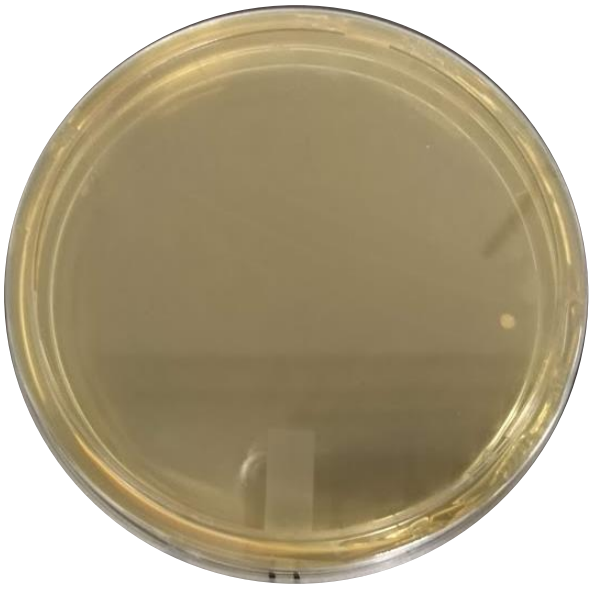

Figure 31: A single colony growing on a YPGalactose + 200 $mg.L^{-1}$ G418 plate after electroporation.

The original electroporation protocol used by Wickes and Edman (1994) recommended the use of 1000 to 5000 ng plasmid DNA for electroporation. Plasmid DNA (2000 ng) and 1000 ng donor DNA were therefore delivered simultaneously into cells using the same electroporation protocol as before. Time constants of 14 to 16 msec were obtained and cells were plated onto YPGalactose plates as before. Four to six colonies were seen on all of the plates, except one of the YPGalactose + 200 $mg.L^{-1}$ G418 plates after 5 days. These colonies were transferred to minimal medium plates lacking adenine or histidine. No colour change (white to red) was observed in colonies growing on plates without adenine and colonies still had the ability to grow on histidine-deficient plates, indicating an intact adenine and histidine synthesis pathways (**Fig. 32**). Gene knockout was therefore unsuccessful.

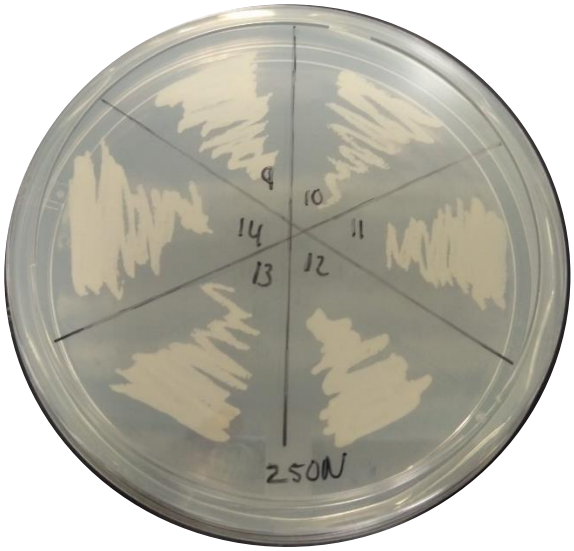

Figure 32: One of the minimal medium plates without histidine and adenine to which transformants were transferred. Growth of white cells indicate that gene targeting was unsuccessful.

Since Lin and co-workers (2018) had success with transient plasmids expressing CRISPR-Cas9 components in both *C. neoformans* and *C. deneoformans*, their protocol with a few modifications to the electroporation parameters was attempted. *Cryptococcus deneoformans* cells were prepared as before for electroporation and were exposed to an electrical current of 2 kV with a resistance of 200 Ω. This was opposed to 0.5 kV and 1000 Ω used before. A time constant of 5 msec was expected. In this case, the cells would be exposed to a higher voltage, but exposed

to the current for a shorter time. The higher voltage could induce more pores in the capsule and cell wall, but could also result in a higher mortality rate. Amounts of 2000 ng plasmid DNA and 1000 ng donor DNA were mixed with cells prior to electroporation. Following electroporation, time constants varied between 3.8 and 4.6 msec, close to the expected 5 msec. Cells were recovered in YPGalactose broth and plated on YPGalactose plates with the appropriate antibiotic as before. Three to eight colonies were seen on the antibiotic plates after 5 days. All of the colonies also showed growth when transferred to minimal media, indicating unsuccessful gene editing.

Circular purified plasmid and linear donor DNA fragments were also delivered into cells with the modified Lin *et al*. (2018) electroporation protocol to see if circular plasmids could be delivered more effectively and remain more stable in the cells. Plasmid DNA (3200 ng) and donor DNA fragment (2000 ng) were added to cells. Time constants of 2 to 3.6 msec were obtained. However, no colonies were seen on the antibiotic plates after 5 days of incubation.

A last attempt at electroporation involved using a protocol developed for *Komagataella phaffii* (formerly known as *Pichia pastoris*) by Wu and Letchworth (2004) (see section 3.4.2 of this chapter). This protocol involves pre-treating the cells with lithium acetate, but otherwise, follow a similar approach to the other electroporation protocols mentioned in this study. Monovalent cations, such as Li^+ from lithium acetate, are known to enhance the efficacy of in-tact yeast cell transformation, most probably because of its chaotropic effect (Kawai *et al*., 2010). Cells were furthermore maintained in a mixture of sorbitol and YPGalactose following electroporation to protect against osmotic stress caused by pore formation in the capsule and cell wall. A strain without the ability to produce a capsule, *C. neoformans* LMPE101, was included in this experiment to test if the capsule plays a significant role in electroporation efficacy. A comparison between *C. deneoformans* UOFS Y-1378 and *C. neoformans* LMPE101 cells can be seen in **Fig. 33**. This protocol recommends lower amounts of DNA to be added to cells and 300 ng plasmid DNA and 600 ng donor DNA were therefore mixed with cells. A voltage of

1.5 kV, a capacitance of 25 µF and resistance of 200 Ω were employed to yield a theoretical time constant of 5. Actual time constants varied between 4.1 and 4.6 msec. No *C. deneoformans* colonies were seen on the antibiotic plates after 5 days and only a single *C. neoformans* LMPE101 colony was seen on a YPD + 100 $mg.L^{-1}$ nourseothricin plate, indicating that the capsule did not play a role in electroporation efficiency, at least with the conditions employed in this protocol.

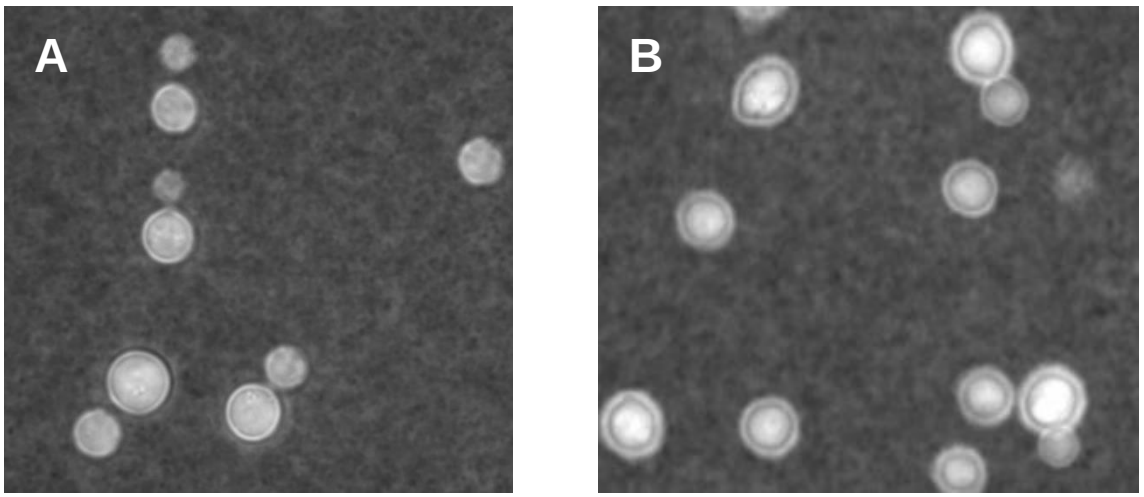

Figure 33: A light micrograph of an India ink stain of the acapsular *C. neoformans* LMPE101 (A) and capsule-producing *C. deneoformans* UOFS Y-1378 strains (B).

4.2.2 Lithium acetate-based chemical transformation

Chemical transformation methods are often used for yeast transformation and Ou and co-workers (2011) used a lithium acetate yeast transduction kit to deliver plasmid DNA into *C. neoformans*. Another approach to delivering the CRISPR-Cas9 components into *C. deneoformans* therefore involved following a lithium acetate-based yeast transformation protocol developed for *C. albicans* by Nguyen and co-workers (2017) described in section 3.4.3 of this chapter. This approach was also unsuccessful, and no colonies were seen on YPGalactose plates containing antibiotics after incubation for 5 days.

5. Conclusions

Transforming microbial cells with plasmid DNA is a routine molecular laboratory technique. A myriad of techniques is available to achieve this in different types of

cells, dictated by how penetrable the cell is. As with plant cells, yeasts of the genus *Cryptococcus* are very impenetrable and are therefore commonly only transformed with two physical transformation techniques, electroporation and biolistic transformation. Previously, homologous integration of a plasmid into the cryptococcal genome was only effective when plasmids were delivered with a biolistic gene gun (Lin *et al.*, 2014). It was proposed that electroporation could not deliver sufficient plasmid into the nucleus for genomic integration in order to obtain stable transformants (Davidson *et al*., 2000), although the plasmid would still need to be transported to the nucleus for expression of selectable markers to obtain transformants in the first place. Lin and co-workers (2014) saw that using a G418 resistance marker seemed to enhance the genomic integration of plasmids delivered with electroporation into *C. neoformans* cells. With the availability of CRISPR-Cas9, researchers do not have to rely on homologous integration alone to disrupt a gene. A gene can now be cleaved and knocked out with the integration of donor DNA provided that the CRISPR-Cas9 components are expressed before their vectors are lost. Electroporation can, therefore, be effectively utilised for gene targeting using CRISPR-Cas9.

This study followed the development of a one-step system with both CRISPR-Cas9 components on a single plasmid to allow for one-step gene targeting as well as to restore the gene in a second round of transformation. Delivering this plasmid and the accompanying donor DNA fragment into *C. deneoformans* cells proved difficult, however. The most significant challenge was the low transformation efficiency obtained with electroporation. Different approaches to optimise electroporation yielded varying results and the best results were obtained when large amounts of plasmid DNA and donor DNA were used. This was the case irrespective of the electroporation parameters used; 0.5 kV, as well as 2 kV, gave more or less the same results when 3200 ng plasmid and 2000 ng donor DNA were mixed with cells. The use of 1.5 kV for electroporation (electroporation/lithium acetate combination) gave no results with less DNA used (300 ng plasmid and 600 ng donor DNA). In contrast, Wu and Letchworth (2004) found that the addition of large amounts of plasmid are counterproductive and yielded fewer *Komagataella phaffii* transformants than when small amounts were added. They found that 10 ng DNA per

transformation reaction gave the most transformants, while 300 ng DNA still gave good results. It is unclear why more DNA resulted in more transformants in this study. No colonies were obtained when circular plasmids were delivered into cells and only a single *C. neoformans* LMPE101 transformant was obtained when a lithium acetate and electroporation combination protocol was employed, even though the electroporation portion remained the same as other electroporation protocols used in this study. From the few colonies obtained from transformation, none remained stable after electroporation and the CRISPR-Cas9 components were not expressed before the plasmids carrying the cassettes were lost, as no phenotypic changes were observed. Using a lithium acetate-based transformation protocol, as is often used for other yeasts, also proved to be ineffective. As a solution, a biolistic gene gun can be used in the future to deliver the constructed plasmids. This equipment was unfortunately unavailable to us during this study. Effective transformation will allow us to target suspected virulence genes, as was the original intent of this study.

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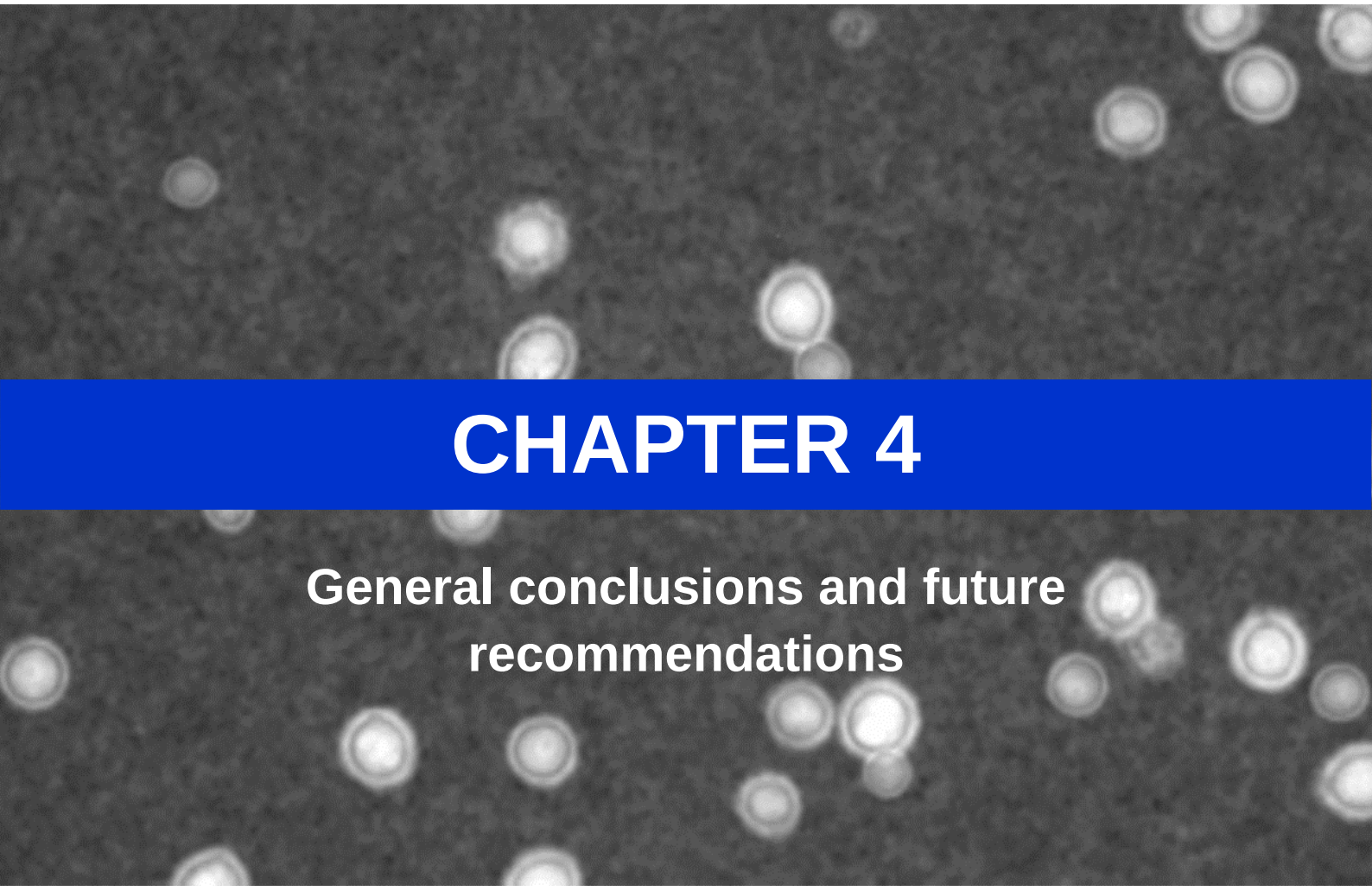

CHAPTER 4

General conclusions and future recommendations

1. General conclusions and future recommendations

The yeasts *Cryptococcus neoformans* and *C. deneoformans* are responsible for the majority of cryptococcal meningoencephalitis cases (Chayakulkeeree & Perfect, 2008). These yeasts are a threat especially to individuals suffering from AIDS. Only a handful of drugs are currently used to treat cryptococcal infections and few are readily available in developing countries were AIDS are widespread (Perfect, 2013; Perfect & Bicanic, 2015). The development of new drugs against cryptococcal infections could, therefore, improve the lives of people suffering from AIDS. New drugs are most often discovered by screening for compounds that show activity against these pathogens. It is however very beneficial to be able to make mutants lacking certain virulence factors to study what effect a potential drug would have on disease if it interferes with the relevant virulence factor. This can only be achieved when genes can be reliably knocked out or knocked down. Until recently, this was only achievable by introducing a synthetic DNA fragment into cells for homologous integration into a specific gene, disrupting gene function and in turn getting rid of the virulence factor. The most reliable technique for delivering such a DNA fragment into cells for genomic integration was with a biolistic gene gun, an expensive piece of equipment. Slightly cheaper methods, such as electroporation, were shown to be ineffective for DNA delivery intended for genomic integration. Lin and co-workers (2014) found that using a G418 resistance marker significantly improved genomic integration of linear plasmids delivered into *C. neoformans* cells with electroporation. However, this was not seen in this study where the G418 marker was compared to a nourseothricin resistance marker. A later study by the same group found that electroporation can be used to introduce plasmids with CRISPR-Cas9 components into cryptococcal cells (Fan & Lin, 2018). The components are expressed, and a targeted gene cleaved before the plasmids carrying the genes are lost.

In this study, an initial approach was to deliver a plasmid carrying a *CAS9* gene into the cells for integration at specific sites in the genome, similar to what Arras and co-workers attempted (2016). We failed to obtain stable transformants expressing the gene and attempted a similar approach as Fan and Lin (2018) and introduced plasmids carrying both CRISPR-Cas9 components into cells with electroporation.

These plasmids were intended to be maintained episomally, with expression of the components before the plasmids are degraded. Electroporation proved to be very ineffective and more than one colony were only obtained when large quantities of DNA were added to cells (3200 ng plasmid DNA and 2000 ng donor DNA). No technique followed in this study yielded more than eight colonies per plate and none of these colonies expressed the CRISPR-Cas9 components. This could be attributed to several reasons. The *C. deneoformans* strain used in this study (UOFS Y-1378) could be less susceptible to electroporation than other *C. neoformans* and *C. deneoformans* strains used by Arras and co-workers (2016) and Fan and Lin (2018). This is however unlikely, as electroporation of the *C. neoformans* LMPE101 strain also only yielded one colony. The amount of DNA used could also be increased, although this might reduce the time that cells are exposed to the electrical current as more salt is introduced, causing a decrease in the resistance of the sample. The cell density of the samples could also be increased. Wu and Letchworth (2004) found that cell densities of $1x10^{10}$ per 80 µL *Komagataella phaffii* cells gave the best results. The cell density per sample used in this study was about $3x10^{8}$ per 40 µL. The amount of DNA and cell density used here were however well within the range used by Fan and Lin (2018) and Wickes and Edman (1994) and this is therefore also an unlikely cause of the low transformation efficiency. Another cause could be the electroporation equipment. Some researchers obtained varying results using electroporators from different manufacturers. Day and Lichtenstein (1992) stated that many factors contribute to successful electroporation and that even trace amounts of ions in water used to make up solutions could have an effect; electroporation should therefore be optimised for every laboratory. Old electroporators could also cause low transformation efficiencies as the electrolytes in the capacitors used to store charge, degrade and change over time (Celaya *et al*., 2011). For this reason, the Bio-Rad Gene Pulser Xcell™ Electroporation System manual recommends that capacitors should be calibrated approximately every one to three months or whenever there is a concern about the accuracy of the capacitors.

Future recommendations therefore include repeating the electroporation protocols with a recently serviced and calibrated electroporator. Transformation should also be attempted with a biolistic gene gun, which was unavailable to us at

the time of this study. Other transformation protocols used for the transformation of cryptococcal yeasts could also be attempted, such as protoplasting and *Agrobacterium*-mediated transformation, although some of these methods might render cells unsuitable for virulence studies in an animal model. When an effective transformation method is found, virulence genes can be removed from these pathogens, as was the original purpose of this study.

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