

Study of *HST6* function in *Candida albicans* through the application of
a CRISPR-Cas9 gene editing system

by

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CHAPTER I

Literature Review

1. Abstract

Candida albicans is an opportunistic fungal pathogen that can cause infections, which may lead to mortalities in immunocompromised patients. This yeast lives as a commensal in various sites of the human body, such as the digestive tract, bloodstream, skin and oral cavity, all with different conditions. For survival of this organism, changes in the surrounding environment need to be monitored and here the Mitogen Activated Protein kinase pathway plays a role in processing these changes. The ATP-binding cassette transporters, which include the *HST6* gene, also play a role in signal transduction in *C. albicans*. *HST6* plays a role in various stress responses, including oxidative, osmotic, cell wall, heavy metal and heat stress. To elucidate the function of genes such as *HST6*, the clustered regularly interspaced short palindromic repeats (CRISPR)–Cas9 system was used. This system makes use of a Cas9 endonuclease and a guide RNA, which directs the Cas9 for double stranded cleaving to edit and eliminate genes. This review will focus on the Mitogen Activated Protein kinase pathway and the ATP-binding cassette transporters in *C. albicans* and an overview will be given on the CRISPR-Cas9 gene editing system. Also, as little is known about signal transduction pathways in *Candida dubliniensis*, a few factors will be discussed.

2. Introduction

Candida albicans forms part of the normal microbiota of healthy humans where it exists as a harmless commensal organism on the skin and in the oral cavity and urogenital and gastrointestinal tracts (Odds, 1988; Homann *et al.*, 2009; Lu *et al.*, 2011; Brown *et al.*, 2014). However, when the immune system becomes compromised, *C. albicans* can cause life-threatening systemic infections (Biswas and Morschhäuser, 2005; Brown *et al.*, 2014). As *C. albicans* can survive as a commensal

in various anatomically distinct sites of the human body, it must be able to adapt to different environmental conditions inside the host (Biswas *et al.*, 2007). Environments such as the bloodstream, mucosa and internal organs give different challenges to the survival of *C. albicans*, such as osmotic, oxidative and enzymatic processes that may be harmful to the cell (Brown and Gow, 1999; Navarro-García *et al.*, 2005). For survival of *C. albicans*, a constant monitoring of the changes in the surrounding environment needs to take place where the MAP (Mitogen-Activated Protein) kinase pathway plays a role in the processing of these external stimuli in eukaryotes (Navarro-García *et al.*, 2005).

The MAP kinase signal transduction pathway functions by sequential protein activation (Navarro-García *et al.*, 2001). Five MAP kinases have been identified in *Saccharomyces cerevisiae* which are allocated to six different MAP kinase pathways namely the mating pheromone response pathway, the pseudohyphal development pathway, the HOG pathway, the protein kinase C (PKC) or cell integrity pathway and the spore wall assembly pathway (Elion, 2000; Herskowitz, 1995; Gustin *et al.*, 1998; Posas *et al.*, 1998; Hohmann, 2002). Less is known about signal transduction pathways in *C. albicans*, but according to Vylkova *et al.* (2007) three MAP kinase pathways have been identified so far in *C. albicans*. These include the cell integrity pathway, the Cek1 pathway and the Hog1 (high-osmolarity glycerol) pathway.

Another signal transduction pathway in *Candida albicans* includes the ATP-binding cassette (ABC) transporters. According to Klein *et al.* (2011), ABC proteins play a role in a variety of stress-related and physiological processes in yeasts. These includes mitochondrial iron homeostasis, ribosomal biogenesis and translation, transport of metabolic intermediates, pheromones and lipids across cellular membranes and drug detoxification. It was also discovered that ABC transporters can lead to multidrug resistance (MDR) in pathogenic yeast strains which counteracts therapy of fungal infections (Klein *et al.*, 2011).

Six subfamilies of ABC transporter proteins are present in *C. albicans* which include the ABCG/PDR, ABCB/MDR, ABCC/MRP, ABCD/ALDP, ABCF/YEF3 and ABCE/RLI subfamilies (Prasad *et al.*, 2015). Several genes belong to each of these subfamilies, with *HST6* belonging to the MDR subfamily and would be specifically focussed on in this review.

HST6 is a functional homologue of Ste6p (present in *S. cerevisiae*) which plays a role in mating pheromone α -factor export at the plasma membrane (Bauer *et al.*, 1999; Kuchler *et al.*, 1993; Kuchler *et al.*, 1989). In a study performed by Motaung (2015) it was discovered that a *C. albicans* deletion mutant ($\Delta\Delta hst6$) was sensitive to oxidative, osmotic, cell wall, heavy metal and heat stress. It was proposed that Hst6p might play a role in the response of *C. albicans* to various stressors and as *HST6* was confirmed to only play a role in mating pheromone export, this was an interesting discovery. This might link *HST6* to the MAP kinase pathway as the Hog1 pathway is involved in osmoadaptation (Brewster *et al.*, 1993), heavy metal stresses (Mains *et al.*, 1990) as well as adaptation to oxidative stress and chlamyospore formation (Alonso-monge *et al.*, 2003).

3. ABC transporters

ABC transporters are an efflux pump gene family that is associated with small molecules moving across the plasma membrane (Michaelis and Berkower, 1995). Higgins (1995) stated that the ABC superfamily is probably the most diverse protein family that functions in mediating the selective solute movement across a biological membrane.

Proteins of the ABC superfamily all contain at least one nucleotide binding domain (NBD) (Prasad *et al.*, 2015). The NBD is the energy source for these proteins and contains highly conserved motifs. These motifs include the Walker A ([AG]-x(4)-G-K-[ST]) and Walker B (D-E-x(5)-D) motifs which are separated by ~ 120 amino acid residues (Walker *et al.*, 1982) and a signature motif (LSGGQ) that lies between the two Walker motifs (Kovalchuk and Driessen, 2010; Schmees *et al.*, 1999). Most of these proteins possess transmembrane domains which are considered ABC transporters.. According to Prasad *et al.* (2015), a one NBD and a one TMD containing protein is a half transporter. A protein that consist of TMD and NBD in duplicate is considered a full transporter.

A common domain organization, shared by all ABC transporters, contains four “core” domains that can be fused together into multidomain proteins or that may be expressed as separate polypeptides (Higgins 1995). The membrane is spanned several times by two transmembrane domains in order to form the pathway through

which solutes can cross the membrane. ATP hydrolysis is coupled to solute movement by two ATP-binding domains that are found at the cytosolic face of the membrane.

Up till recently *C. albicans* had 28 putative ABC proteins which include 12 half transporters that remain uncharacterized (Gaur *et al.*, 2005). These 28 genes were divided into six subfamilies, which include the *PDR* (pleiotropic drug resistance) gene family, the *MDR* (multidrug resistance) gene family, the *MRP* (multidrug resistance-associated protein) gene family, the *RLI* (RNase L inhibitor)/*ALDP* (adrenoleukodystrophy protein) gene family, the *YEF3* (yeast elongation factor -3) gene family and a sixth “others” group. Recently a rearrangement has been made and according to Prasad *et al.* (2015), 26 ABC proteins exist with 19 of them being ABC transporter proteins. These six subfamilies include the ABCG/PDR, ABCB/MDR, ABCC/MRP, ABCD/ALDP, ABCF/YEF3 and ABCE/RLI subfamilies.

3.1 The ABCG/PDR gene family

The *PDR* gene family consists of nine members, which includes the *CDR* (Candida drug resistance) genes (*CDR1* and *CDR2*) (Prasad *et al.*, 1995; Sanglard *et al.*, 1997), *CDR3* (Balan *et al.*, 1997), *CDR4* (Franz *et al.*, 1998), *CDR11* (Ca918, assembly #20 <http://www.candidagenome.org/download/Assembly20notes/>), *SNQ2*, orf19.4531, *ADPI* and orf19.3120 (Prasad *et al.*, 2015).

3.1.1 *CDR1* and *CDR2*

CDR1 and *CDR2* are full ABC transporters involved in antifungal drug resistance and encode Cdr1p and Cdr2p which are plasma membrane ABC-type transporters (Holmes *et al.*, 2008; De Micheli *et al.*, 2002; Prasad *et al.*, 2015). *CDR1* codes for a 1501-amino acid protein (Cdr1p) and *CDR2* for a 1499-amino acid protein (Cdr2p) (Klein *et al.*, 2011).

In a study performed by Coste *et al.* (2004) it was discovered that *CDR1* and *CDR2* can be upregulated in *C. albicans* by exposing cells to drugs such as fluphenazine. It can also be upregulated developing resistance to azoles. In other words,azole resistant *C. albicans* isolates show overexpression of Cdr1p (Prasad and Rawal, 2014). The *ERG11* (ergosterol 11) gene in *C. albicans* encodes a target protein whose inhibition becomes reduced when multiple transporter genes become

upregulated. This upregulation leads to the enhanced efflux of azoles which results in a decrease of drug accumulation as well as the reduced inhibition (Perea *et al.*, 2002; Sanglard *et al.*, 1997; Sanglard *et al.*, 1995; White, 1997). According to Holmes *et al.* (2008) Cdr1p expression contributes more to fluconazole (FLC) resistance than what Cdrp2 does.

In-to-out transbilayer phospholipid movement is carried out by *CDR1* and *CDR2* and is energy-dependent (Smriti *et al.*, 2002). It is also sensitive to cytochalasin E (a cytoskeleton disrupting agent) and N-ethylmaleimide (a sulphhydryl blocking agent). According to Smriti *et al.* (2002) *CDR1* and *CDR2* are ABC transporters that are phospholipid translocators of *C. albicans* and Dogra *et al.* (1999) showed how aminophospholipid translocation could be mediated by *CDR1*.

3.1.2 CDR3

According to Balan *et al.* (1997), *CDR3* codes for a 1501-amino acid protein. The structure of this full ABC transporter, Cdr3p, consists of two halves that each contains an N-terminal hydrophilic domain with consensus ATP binding sequences. It also contains a C-terminal domain that is hydrophobic with six predicted transmembrane segments. Cdr3p is highly homologous to Cdr1p and Cdr2p (56 and 55% amino acid sequence each) and the expression of *CDR3* is coupled to white-opaque switching of *C. albicans* (Balan *et al.*, 1997).

Unlike *CDR1* and *CDR2*, *CDR3* is not involved in drug resistance (Smriti *et al.*, 2002). Out-to-in translocation of phospholipids is carried out between the two plasma membrane monolayers by the action of *CDR3*. This translocation is energy dependent and it is insensitive to cytochalasin E and N-ethylmaleimide. According to Smriti *et al.* (2002) Cdr3 is an ABC transporter that is a phospholipid translocator of *C. albicans*.

3.1.3 CDR4

Cdr4p, which is encoded by *CDR4*, is a full ABC transporter that consists of 1490 amino acids and was discovered to be highly homologous to Cdr1, Cdr2 and Cdr3 (Franz *et al.*, 1998). Franz *et al.* (1998) stated that it does not seem that *CDR4* play a role in fluconazole resistance as a *C. albicans* mutant, which does not contain

one of the *CDR4* copies, was not hyper-susceptible as compared to the parent strain. According to Prasad *et al.* (2015) *Cdr4* is a full ABC transporter that is involved in lipid translocation.

3.1.4 Other ABCG/PDR members

According to the *Candida* Genome Database, *Cdr11* is a full ABC transporter that is merged with *orf19.919* and consists of 1512 amino acids, *Snq2* is also a full ABC transporter that is similar to *S. cerevisiae* *Snq2* and it consists of 1495 amino acids (Prasad *et al.*, 2015). *Orf19.4531* consists of 1274 amino acids and is an ABC transporter that is similar to *S. cerevisiae* *YOL075C*. *ADP1* consists of 1038 amino acids and is an ABC transporter that is similar to *S. cerevisiae* *Adp1* (Prasad *et al.*, 2015). *Orf19.3120* is an ABC transporter that is similar to *S. cerevisiae* *YOL075C* and consists of 579 amino acids (Prasad *et al.*, 2015).

3.2 The ABCB/MDR gene family

According to Raymond *et al.* (1998) *HST6* codes for a 1323-amino acid protein. This protein contains an intramolecular duplicated structure of which each repeated half contains six potential hydrophobic transmembrane segments. It also contains a hydrophilic domain with consensus sequences for an ATP-binding fold. *HST6* is a functional homologue of the *S. cerevisiae* *Ste6p* which plays a role in mating pheromone α -factor export at the plasma membrane (Bauer *et al.*, 1999; Kuchler *et al.*, 1993; Kuchler *et al.*, 1989).

According to the *Candida* Genome Database *Atm1*, *Mdl1* and *Mdl2* are half ABC transporters. *Atm1* is similar to the *S. cerevisiae* *Atm1* and consists of 750 amino acids, *MDL1* is similar to the *S. cerevisiae* *MDL1* and consists of 685 amino acids and *MDL2* is similar to *S. cerevisiae* *MDL2* and consists of 783 amino acids (Prasad *et al.*, 2015).

3.3 The ABCC/MRP gene family

This gene family consists of four members. *YOR1*, *BPT1* and *YCF1* are full ABC transporters consisting of 1488, 1490 and 1580 amino acids respectively. *MLT1* on the other hand is a full vacuolar ABC transporter that is involved in phosphatidylcholine import and it consists of 1606 amino acids (Prasad *et al.*, 2015).

3.4 The ABCD/ALDP gene family

3.4.1 PXA1

According to the *Candida* Genome Database *PXA1* is a half ABC transporter that is similar to *S. cerevisiae* Pxa1 and it consists of 768 amino acids (Prasad *et al.*, 2015).

3.4.2 PXA2

According to the *Candida* Genome Database *PXA2* is a half ABC transporter that is similar to *S. cerevisiae* Pxa2 and consists of 667 amino acids (Prasad *et al.*, 2015).

3.5 The ABCF/YEF3 gene family

3.5.1 KRE30

According to Prasad *et al.* (2015) *KRE30* is an ABC non-transporter and mutations lead to hypersensitivity to amphotericin B and it consist of 609 amino acids.

3.5.2 CEF3

According to the *Candida* Genome Database *CEF3* is a ABC non-transporter and it consists of 1050 amino acids (Prasad *et al.*, 2015).

3.5.3 GCN20

According to the *Candida* Genome Database *GCN20* is a ABC non-transporter protein that is similar to *S. cerevisiae* Gcn20 and it consists of 751 amino acids (Prasad *et al.*, 2015).

3.5.4 ELF1

According to the *Candida* Genome Database *ELF1* is a ABC non-transporter of 1195 amino acids (Prasad *et al.*, 2015).

3.6 The ABCE/RLI gene family

This gene family have only one representative, *RLI1*, and according to the *Candida* Genome Database it is a ABC non-transporter that is similar to *S. cerevisiae* *RLI1* and it consists of 622 amino acids (Prasad *et al.*, 2015).

3.7 The “others” family

According to the *Candida* Genome Database, *MODF* and *CAF16* are ABC nontransporters. *MODF* is similar to *S. cerevisiae* YDR061w and consists of 545 amino acids where *CAF16* is similar to *S. cerevisiae* YFL028c and consists of 320 amino acids (Prasad *et al.*, 2015).

4. Mitogen-Activated Protein Kinase (MAP Kinase) Pathway

MAP kinase pathways are responsible for monitoring changes that take place in the surrounding environment of *Candida albicans* and other eukaryotes and they play a role in processing these external stimuli (Navarro-García *et al.*, 2005; de Dios *et al.*, 2010). According to Kültz and Burg (1998) three major subgroups of MAP kinases exists which include the MAPK3 subgroup, the stress-activated protein kinases (SAPKs) and the extracellular-signal-regulated kinases (ERKs).

4.1 MAP kinase pathway activation

MAP kinases are activated by the simultaneous phosphorylation of threonine and tyrosine residues (Nishida and Gotoh, 1993; Gartner *et al.*, 1992). This occurs by the action of MAP kinase kinases (MAPKKs) that have a dual specificity for both tyrosine and serine/threonine which are phosphorylated by the MAP kinase kinase kinases (MAPKKKs). The MAPKKs are turned on by the phosphorylation of serine/threonine that is catalysed by an immediate kinase that lies upstream (Nishida and Gotoh, 1993). Transcription factors and the increase in defence target genes are being sequentially activated through the activation of these pathways (de Dios *et al.*, 2010).

4.2 Different MAP kinase pathways in *Candida albicans*

4.2.1 The cell integrity pathway

The cell integrity pathway is known to be activated by cell wall stress and serves a role in responses to hypo-osmotic and oxidative stress, antifungal drugs, biofilm development and invasive hyphal growth (Navarro-García *et al.*, 2005; Kumamoto, 2005). This pathway is mediated by the Mkc MAP kinase, which belongs to the ERK subgroup (Kumamoto, 2005; Navarro-García *et al.*, 2005; de Dios *et al.*, 2010), and Navarro-García *et al.* (2005) also determined that Pkc1 (a yeast homolog of protein kinase C) is responsible for the phosphorylation of Mkc1p upon either a hyperosmotic challenge or an oxidative challenge. It was also found that both Pkc1p and Hog1p controls Mkc1p activation and that both, Mkc1p and Pkc1p, are necessary to respond appropriately to cell wall injuries. It was speculated by Brown *et al.* (2014) that Bck1 is a MAPKKK and that Mkk1 is a MAPKK in the cell integrity pathway of *C. albicans*. In this way Bck1 phosphorylates Mkk1 which in turn activates Mkc1p by phosphorylation together with Pkc1p.

4.2.2 The Cek1 pathway

Arana *et al.* (2007) mentioned four different MAP kinase pathways in *C. albicans*. However, according to Vylkova *et al.* (2007) only three MAP kinase pathways have been identified in *C. albicans*. The confusion exists because the Cek1 pathway is mediated by the Cek1 and Cek2 MAP kinases, therefore there are two MAP kinases that functions in one pathway. The Cek1 pathway is involved in cell wall biogenesis, virulence, and mediates filamentation and mating (Leberer *et al.*, 1996; Eisman *et al.*, 2006; Monge *et al.*, 2006). Hst7 plays a role in the Cek1 pathway where it acts as a MAP kinase kinase (Leberer *et al.*, 1996; Monge *et al.*, 2006) and according to Brown *et al.* (2014) Ste11 serves the role of the MAP kinase kinase kinase in this pathway. *CEK1* plays a role in cell wall construction, growth and invasive growth (Arana *et al.*, 2007) and it was shown that the deletion of *CEK1* had a reducing influence on mating (Chen *et al.*, 2002). It was observed that when both *CEK1* and *CEK2* were deleted, the mutant was unable to mate (Chen *et al.*, 2002). Therefore Cek2

acts as the MAP kinase in mating response in this pathway (Chen *et al.*, 2002; Brown *et al.*, 2014). Deletion of *CEK1* also showed reduced virulence and defects in hypha formation (Csank *et al.*, 1998).

4.2.3 The Hog1 pathway

The Hog1 pathway which is probably the most studied MAP kinase pathway in *C. albicans* (Navarro-García *et al.*, 2005), is involved in osmoadaptation (Brewster *et al.*, 1993), heavy metal stresses (Mains *et al.*, 1990) as well as adaptation to oxidative stress and chlamyospore formation (Alonso-monge *et al.*, 2003). Hog1 belongs to the SAPKs subfamily (Kültz and Burg 1998) and it was also suggested that Hog1 has a regulatory function in the cell integrity MAP kinase (Mkc) and the Cek1 MAP kinases (Cek1 and Cek2) (Eisman *et al.*, 2006; Román *et al.*, 2005). Pbs2 is a MAP kinase kinase (MAPKK) which activates Hog1 (Arana *et al.*, 2007; Cheetham *et al.*, 2007). Pbs2 is activated by Ssk2, which is a single MAP kinase kinase kinase (MAPKKK). Chauhan *et al.* (2003) explained that Hog1 becomes phosphorylated when Ssk1 is involved. This is a response regulator protein that uses Ssk2p (MAPKKK) to activate the HOG pathway (Arana *et al.*, 2007).

5. *Candida dubliniensis*

Candida dubliniensis, which is closely related to *Candida albicans* (Sullivan *et al.*, 1997), was discovered to be associated with oral candidosis in patients infected with the human immunodeficiency virus (HIV) and those with Acquired ImmunoDeficiency Syndrome (AIDS) (Moran *et al.*, 1998). According to Sullivan *et al.* (1993) *C. dubliniensis* was first detected between 1991 and 1992 in a dental hospital in Dublin, therefore less is known about this species of *Candida* as this was a more recent discovery than *C. albicans*. Several studies need to be performed on this species to gather information about the signal transduction pathways of *C. dubliniensis*. In a study performed by Moran *et al.* (1998) a few homologs of *CDR1*, *CDR2* and *MDR1* genes of *C. albicans* were found to be present in *C. dubliniensis*, for example *CdMDR1* (*Candida dubliniensis MDR*), which is highly homologous to *CaMDR1* (*Candida albicans MDR*). *MDR1* was also the first complete protein-encoding ORF to be

determined from *C. dubliniensis* (Moran *et al.*, 1998). Moran *et al.* (1998) obtained results which indicated that *C. dubliniensis* encodes multidrug transporters that are capable of mediating resistance to fluconazole. According to Pinjon *et al.* (2003) overexpression of *CdMDR1* contributes to resistance in fluconazole-resistant isolates of *C. dubliniensis*.

6. CRISPR-Cas9

The clustered regularly interspaced short palindromic repeats (CRISPR)–Cas9 system for gene editing is based on the function of the Cas9 endonuclease and the single-guide RNA (sgRNA) (Charpentier 2015). The sgRNA directs Cas9 to create double-stranded breaks (DSBs) at a target locus (Doudna and Charpentier 2014). The Cas9 cuts the genome of the organism at regions which bind to the 20-bp guide from the sgRNA, if followed by the protospacer adjacent motif (PAM) which consists of the NGG (where N is any nucleobase followed by two guanine nucleobases) sequence (Vyas *et al.*, 2015). These double-stranded breaks must be repaired in the genome as they are lethal to the cell (Nguyen *et al.*, 2017). This can be done via non-homologous end joining (NHEJ) or homology-directed repair (HDR). NHEJ is error prone and happens spontaneously when a DSB occurs in the genome of the organism. HDR can be used to insert modifications (large pieces of heterologous DNA or single-base differences) at the target locus as donor DNA or a DNA repair template, thus modifying the genome of the organism.

The CRISPR-Cas9 system was first observed in *Escherichia coli* in 1987 as repeats of short segments in the genome with spacers in between the repeats (Ishino *et al.*, 1987). It was stated that the Short Regularly Spaced Repeats (SRSRs) are palindromic sequences, approximately 24-40 base pairs (bp) in size, with repeats up to approximately 11 bp, in length (Regularly *et al.*, 2000). Jansen *et al.* (2002) studied this family of repetitive DNA sequences present in both achaea and bacteria and discovered that it is characterized by direct repeats (from 21 to 37 bp). Similar sized non-repetitive sequences were found between the direct repeats and this family was referred to as the clustered regularly interspaced short palindromic repeats (CRISPR). In 2005 it was suggested that these repeats in prokaryotes (found in Bacteria and Achaea) originated from past invasions and protects the cell from future invasions as they provide immunity to the organism (Bolotin *et al.*, 2005). It was shown that the

spacers were derived from sequences that were previously observed in the cell, such as from bacteriophages and plasmids (transmissible genetic elements) or chromosomes (Soria 2005). CRISPR-associated (Cas) protein families were identified (Cas1 – Cas4) and was found to occur near a repeat cluster (Haft *et al.*, 2005). In 2006 approximately 25 distinct protein families which contain the protein sequences of several Cas gene products were classified (Makarova *et al.*, 2006). It was hypothesized that the inserts of CRISPR function as prokaryotic siRNAs and that it is a method of defence against plasmids and phages which yields heritable immunity, even though very unstable. Barrangou *et al.* (2007) performed experiments with *Streptococcus thermophilus* and lytic phages and stated that CRISPR and the Cas genes are responsible for providing resistance against phages and that the specificity is in correlation with the spacer-phage sequence similarity. Barrangou and Horvath (2012) showed how the CRISPR-Cas system can be used in an immunization process by inserting short sequences of virulent phages into a CRISPR site which are formed into small interfering RNAs. This is guided by a protein complex which cleaves matching foreign DNA. In 2013 Sun *et al.* cultured *Streptococcus thermophilus* and observed that after phage exposure, spacers yielded genetically diverse populations and that phage mutations were found in the proto-spacer adjacent motif (PAM) or near the end of the PAM site (Sun *et al.*, 2013). It was explained how bacteria and archaea have adaptive immune systems (CRISPR-Cas systems) that attack viruses and plasmids (Oost *et al.*, 2014). The integration of short fragments of these foreign DNA into specific regions (CRISPR loci) leads to immunity when the transcription of these loci yields CRISPR RNAs (crRNAs). This guides the Cas proteins to interfere in the target when the invading nucleic acid is complementary.

In 2011 Deltcheva *et al.* performed experiments on *Streptococcus pyogenes* which revealed tracrRNA and how it directs maturation of crRNAs through the activity of RNase III and the Cas9 protein (then known as Csn1). It was shown how the crRNA, which is base-paired to tracrRNA, forms a structure (sgRNA) which directs Cas9 to induce double-stranded breaks in DNA (Jinek *et al.*, 2012). This discovery led to the potential for gene editing as it can be used to direct the Cas9 to cut a target region that's next to the PAM site. Cas9 together with sgRNA can be used to alter DNA and disruptions of several genes were reported.

The first CRISPR-Cas gene editing system for *Candida albicans* was developed by Vyas *et al.* (2015), using the CRISPR-Cas9 system, as the double deletion mutant yields a red phenotype. They removed both copies of the *ADE2* gene and later also targeted *CDR1* and *CDR2*. Nguyen *et al.* (2017) developed an optimized CRISPR-based genome editing system for *C. albicans* and targeted the *WOR1*, *WOR2*, and *CZF1* genes. Several genes were edited by use of the CRISPR-Cas9 system and the purpose of this project would be to create a CRISPR-Cas9 system to modify genes in *C. albicans* including *HST6*.

7. Conclusion

This review discussed *Candida albicans* and that it forms part of the normal microbiota of healthy human beings and monitors changes in its environment by using the Mitogen Activated Protein (MAP) Kinase Pathway and the ATP-binding cassette (ABC) transporters (Navarro-García *et al.*, 2005; Klein *et al.*, 2011). *HST6* forms part of the ABC transporters and was proposed to play a role in various stress responses in *C. albicans* (Bauer *et al.*, 1999; Kuchler *et al.*, 1993; Kuchler *et al.*, 1989). Stress responses, such as heat stress, osmotic stress, heavy metal stress, oxidative stress and cell wall stress was observed in a PhD thesis by Motaung (2015). By gene removal using the CRISPR-Cas9 system, the function of *HST6*, and other genes, can be determined (Vyas *et al.*, 2015). This system uses an endonuclease (Cas9) that cuts target DNA and a guide RNA that directs the endonuclease for cutting. Double stranded breaks are introduced and the gene can be removed, edited or added back. This system opened the door for gene deletion and will allow for more research to be done on ABC transporters, the MAP Kinase Pathway and *HST6*. By creating mutants (single deletion and double deletion) and complemented strains (where a gene is added back into the genome of the organism) will make room for comparison and elucidation of the function of genes of which little is still known. More research needs to be done on *C. dubliniensis* and by using the CRISPR-Cas9 system, the function of more signal transduction pathways can be determined.

8. Aims and objectives of this study

Aim:

Study the role of *HST6* in *C. albicans* and determine its role in resistance/sensitivity to stress conditions and antifungal drugs

Objectives:

- Conducting an extensive literature survey regarding signal transduction pathways in *C. albicans*
- Construction of a homozygous deletion mutant of *HST6* in *C. albicans* by using the CRISPR-Cas9 System
- Construction of a strain where *HST6* is re-introduced in the deletion mutants (add back)
- Determine the effect of fluconazole on $\Delta\Delta hst6$

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Chapter 2

Construction of a *HST6* homozygous mutant using the *SAT1* flipper system

1. Abstract

Candida albicans is a diploid organism, which requires two rounds of excision/integration in order to create a double deletion mutant, as both alleles of a specific gene, needs to be disrupted. *HST6* forms part of the ATP binding cassette family of transporters and in a previous study was discovered that deletion of both copies leads to mutants showing sensitivity to 93% of stress conditions tested. Using the *SAT1* flipper system, $\Delta\Delta hst6$ was created previously, however a complemented strain was required where the *HST6* gene was reintroduced into the mutant. We aimed to create $\Delta\Delta hst6$ mutants of *C. albicans* in order to create complemented strains, but were unsuccessful using the previously constructed plasmid (pGem-T Easy Hst6::*SAT1* flipper) which contains the *SAT1* flipper system. Integration of the *SAT1* flipper system into the genome of the organisms was unsuccessful, although several transformation protocols were performed, and various factors were changed.

2. Introduction

Candida albicans is a harmless commensal organism that lives in the human body. However, when the immune system becomes compromised, it can cause life-threatening systemic infections (Odds 1988). This organism is diploid, which requires that two rounds of excision/integration must be performed to create double deletion mutants of a certain gene (Homann *et al.*, 2009; Jones *et al.*, 2004). Various methods exist for gene disruption, including the URA blaster method (Fonzi and Irwin 1993), the PCR-based URA blaster method (Wilson *et al.*, 2000), the UAU1 method (Enloe *et al.*, 2000), the URA flipper method (Morschhauser *et al.*, 1999), the MPA^R flipper method (Wirsching *et al.*, 2000), the *SAT1* flipper method (Reuss *et al.*, 2004), the

Cre-loxP flipper method (Dennison *et al.*, 2005), the Clox flipper method (Shahana *et al.*, 2014) and the CRISPR method (Vyas *et al.*, 2015). Some of these methods, especially the ones using auxotrophic markers, affect virulence in *C. albicans* (Lay *et al.*, 1998; Staab and Sundstrom, 2003; Brand *et al.*, 2004). Therefore, the *SAT1* flipper method is the gene disruption tool of choice for knocking out *HST6* in *C. albicans*. This method relies upon the use of a dominant marker (Nourseothricin) and not an auxotrophic marker such as, for instance, in the case of the URA blaster method (Fonzi and Irwin, 1993; Reuss *et al.*, 2004) and it is also possible to remove the marker following selection of a targeted gene.

The *SAT1* flipper system contains a dominant nourseothricin (NTC) resistance marker, which selects for integration of the *Streptomycin acetyltransferase1* (*SAT1*) gene into the genome of the organism (Reuss *et al.*, 2004). Two rounds of excision/integration are necessary to knock out both copies of the gene and therefore to create homozygous mutants. The flipase (*FLP*-mediated) recyclable marker system allows for regeneration of the NTC resistance marker in order for both copies of the gene to be deleted in the second round of integration. The *FLP* gene is flanked by *FLP* target sequences of the genome of *C. albicans*. By growing the organism on maltose, the expression of the *FLP* gene is activated as it is under the control of the regulatable maltose promoter. The deletion cassette between the *FLP* recombinase recognition target sequences is excised, and both copies of the gene are deleted after two rounds of integration.

HST6 in *C. albicans* is a functional homologue of Ste6p which is present in *Saccharomyces cerevisiae* (Bauer *et al.*, 1999; Kuchler *et al.*, 1993; Kuchler *et al.*, 1989). *HST6* also forms part of the ATP binding cassette family of transporter proteins (Prasad *et al.*, 2015). According to Klein *et al.* (2011) ABC transporters can lead to drug resistance in pathogenic yeasts such as *C. albicans* and in order to confirm that *HST6* might play a role in resistance to fluconazole, both a deletion of *HST6* as well as a complemented strain must be created. The aim of this part of the study was to use the *SAT1* flipper to construct a double deletion mutant of *HST6* in *C. albicans*.

3. Materials and Methods

3.1 Strains used

Table 1. *Candida albicans* strains used in this study

Strain number	Genotype
NRRL-Y-27077	Wild type
SC 5314	Wild type
CBS8758-2833	Δ hst6; <i>HST6</i>

3.2 PCR (Polymerase Chain Reaction)

3.2.1 Primers used in this study

Table 2. Primers used in this study

Primer name	Primer sequence (5'-3')
Ca_4F + <i>Nco</i> I	GTGGTGGCTTTCCTACGATAA
Ca_HST6-2R	CCGAACCAATCAAATCTCCAGAAGGT

3.2.2 Linearization of pGem-T Easy Hst6::SAT flipper

A PCR was performed using a previously constructed pGem-T Easy Hst6::SAT flipper plasmid to amplify the region containing 500 bp (base pairs) up and downstream of the *HST6* ORF with the *SAT1* flipper gene in between these regions. This amplification would yield a linear fragment that would be transformed into the *C. albicans* to delete the *HST6* ORF.

The following primers were used: Ca_4F + *Nco*I and Ca_HST6-2R with KOD Hot Start DNA Polymerase (Novagen) to amplify the DNA sequences. The following conditions were used: One cycle of a 2-minute denaturation at 94°C followed by 25 cycles consisting of a 20 second denaturation at 95°C, 10 seconds annealing at 60°C and a 2 minute and 30 seconds elongation at 70°C. This was followed by one final elongation cycle for 7 minutes at 72°C.

3.3 Transformations

3.3.1 Transformation of *C. albicans* by Electroporation

The method published by Kohler *et al.* (1997), was used for electroporation. Cells were diluted 10^{-4} from an YPD (10 g.l⁻¹ Yeast Extract Powder, 20 g.l⁻¹ Peptone Powder, 20 g.l⁻¹ Glucose) preculture in 50 ml fresh YPD broth and incubated overnight at 30°C to an optical density (OD₆₀₀) of 1.6-2.2. Cells were harvested by centrifugation at 5000 x *g* at room temperature for 5 min and the supernatant was discarded. The pellet was resuspended in 8 ml ddH₂O (double distilled H₂O). A volume of 1 ml 10x TE buffer (100 mM Tris-HCl, 10 mM EDTA, pH 7.5) and 1 ml of 1 M lithium acetate were added. The suspension was incubated on a rotary shaker at 150 rpm for 1 h at 30°C. A volume of 250 µl of 1 M dithiothreitol was added and incubated in a rotary shaker at 150 rpm for 30 min at 30°C. Ice-cold ddH₂O (40 ml) was added and cells were harvested by centrifugation at 3300 x *g* for 5 min at 4°C (all steps were performed on ice from here on). The supernatant was discarded, and sequential centrifugation was performed with added 25 ml ice-cold dH₂O and then 5 ml ice-cold 1 M sorbitol. The supernatant was discarded, and cells were resuspended in 50 µl ice-cold 1 M sorbitol. Three microliters the linear PCR amplified fragment (section 3.2.2) were added to a cuvette as well as 40 µl cell mixture and 3 µl carrier DNA (salmon sperm (SS)). Electroporation was carried out in a Bio-Rad Gene Pulser[®] electroporator (0.2 cm cuvette, 1.8 kV). In cases where the kV used was too high (indicated by a spark) conditions were changed (450 µl ice-cold sorbitol were added to the cells and 80 µl of cells were added to the cuvette, electroporation was carried out at 1.4 kV). Following electroporation, cells were washed in 1 ml of ice cold 1 M sorbitol and harvested by centrifugation at 450 x *g* for 5 min at 4°C and resuspended in 1 ml YPD. Cells were incubated at 30°C with shaking for 4 h, centrifuged at 450 x *g* for 5 min at 4°C, the supernatant discarded and resuspended in 100 µl sorbitol. Cells were plated on YPD containing 200 µg.ml⁻¹ NTC (Nourseothricin) and incubated for 2 – 3 days at 30°C.

3.3.2 Transformation of *C. albicans* using Lithium Acetate (LiAc)

The method published by Gietz *et al.* (1992) was used. Competent yeast cells were prepared as follows:

Cells were cultivated on YPD medium and incubated overnight at 37°C. Colonies were picked from the plate and inoculated in 5 ml YPD and incubated overnight with shaking at 37°C. Fifty microliters of culture were inoculated into 5 ml YPD and incubated with shaking at 37°C to an OD₆₀₀ of 0.5-1.0. Cells were harvested by centrifugation for 5 min at 5000 x *g* at room temperature (RT) and the supernatant discarded. Cells were washed with 1 ml ddH₂O and resuspended in 1 ml 1x TE/LiAc (prepared from 10X TE stock solution (100 mM Tris-HCl, 10 mM EDTA, pH 7.5 and filter sterilized) and 10X LiAc stock solution (2 M lithium acetate, pH 7.5 with acetic acid and filter sterilized) and kept on ice.

For the transformation:

Five microliters of SS (denatured at 99°C for 10 min) and 5 µl linear transforming DNA was added to a 1.5 ml microcentrifuge tube. Competent cells (100 µl) were added to the DNA solution and mixed with repeated pipetting. Three hundred microliters of PEG 40% (2.4 ml 50% PEG, 300 µl 10x TE, 300 µl 10x LiAc), pre-warmed at 42°C, was added to the transforming DNA solution and mixed by repeated pipetting. The suspension was incubated stationary at 30°C for 30 min and was heat shocked at 42°C for 15 min. Cells were then harvested by centrifugation for 4 min at 4000 x *g* at RT and the supernatant discarded. Cells were washed with 1 M sorbitol, resuspended in 1 ml YPD and incubated for 24 h at 30°C to express the *SAT1* gene. Cells were washed once with and resuspended in 200 µl 1 M sorbitol. 100 µl of the cell suspension was plated out on YPD plates containing 100 µg.ml⁻¹ NTC and incubated for 2-3 days at 30°C (Reuss *et al.*, 2004; Ramon and Fonzi, 2009). When growth was observed, cells were streaked on YPD plates containing 200 µg.ml⁻¹ NTC.

4. Results and Discussions

A plasmid (pGem-T Easy Hst6::*SAT* flipper) that contained 500 bp upstream and downstream of the *HST6* ORF (open reading frame) with the *SAT1* flipper gene in the place of the *HST6* ORF, was used in this study (Motaung, 2015; Figure 1). This is based on the principle that the *SAT1* flipper gene would flip out the *HST6* ORF after

transformation and would be inserted into the gDNA of the organism in order to create a mutant that contains no copies of the *HST6* ORF after sequential deletion.

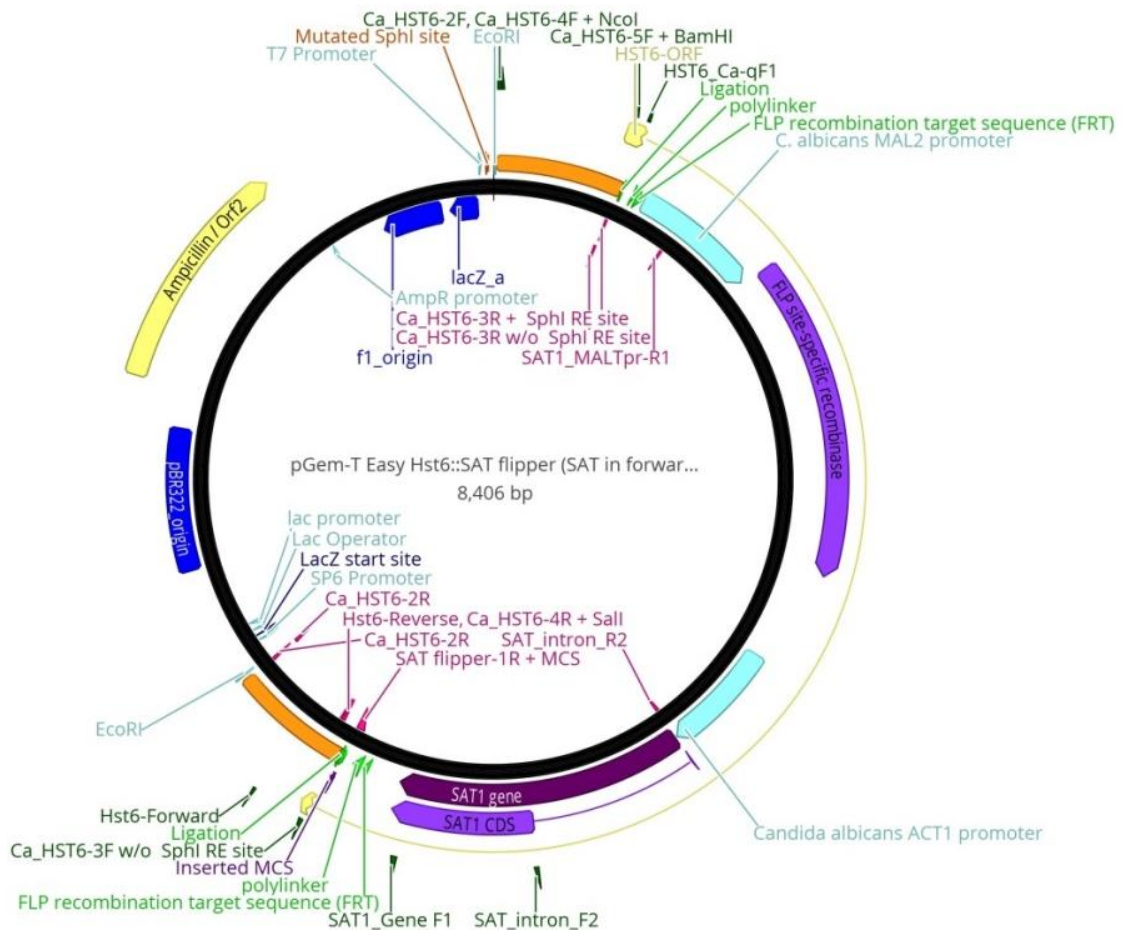


Figure 1 - pGem-T Easy Hst6::SAT flipper plasmid. Figure depicts the deletion construct containing the 500 bp up and downstream regions of *HST6* (orange) flanking the complete *SAT1* Flipper. After PCR, this linear fragment was used to delete one copy of the *HST6* gene and replace the gene with the *SAT1* fragment (Motaung, 2015).

4.1 PCR

PCR was performed using the pGem-T Easy Hst6::SAT flipper plasmid to amplify the region containing 500 bp up and downstream of the *HST6* ORF and the *SAT1* flipper gene in between that. This amplification yielded a linear fragment, that was visualised

using gel electrophoresis (0.8% agarose gel) at 90 V for 30 min, and that would be transformed into the organism to flip out the *HST6* ORF (Figure 2).

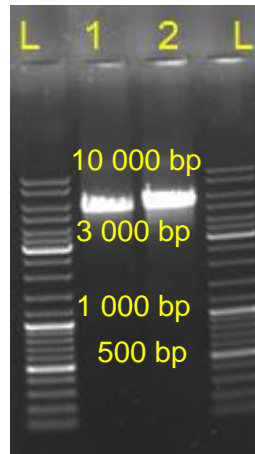


Figure 2 – PCR results. A band containing approximately 5385 bp was observed when primers: Ca_4F + NcoI and Ca_HST6-2R were used to amplify a region of the plasmid DNA.

4.2 Transformations

In a previous study (Motaung, 2015) a heterologous deletion of *HST6* was constructed in *Candida albicans*. This strain (CBS8758-2833) as well as two wild type strains (NRRL-Y-27077 and SC 5314) were used in an attempt to construct a homozygous deletion mutant for *HST6*.

4.2.1 Transformation of *C. albicans* by Electroporation

Following transformation using electroporation, no colonies were observed after 3 days of incubation. This experiment was repeated a number of times without obtaining any transformants. Various changes in transformation conditions were also included in an attempt to obtain successful transformation. This optimization included different concentration of single stranded carrier DNA (SS), omission of SS, amount of sorbitol, different concentrations of cells as well as changes in the electroporation volts.

4.2.2 Transformation of *C. albicans* using LiAc

Growth was observed on negative control plates although transformation was repeated numerous times with different conditions (different incubation temperatures, with or without SS, new PCR product – higher concentration of DNA, incubated with shaking, etc). It seemed that the negative control plate contained more colonies than those of the transformed cells. If transformed colonies were plated on YPD plates containing 150 or 200 $\mu\text{g}\cdot\text{ml}^{-1}$ NTC, no growth was observed, except where growth was observed for one colony on 200 $\mu\text{g}\cdot\text{ml}^{-1}$ NTC plates.

Single colonies were selected and gDNA was extracted (ZR Fungal/Bacterial DNA miniprep) for confirmation (Figure 3) and a PCR was performed using Ca_4F + NcoI and Ca_HST6-2R (Figure 4).

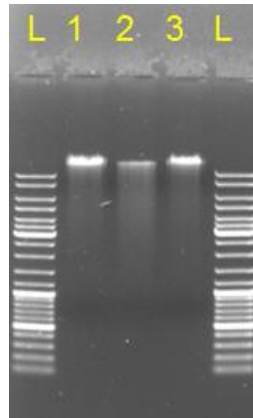


Figure 3 - gDNA extraction. Lane 1-3 indicates the extracted genomic DNA

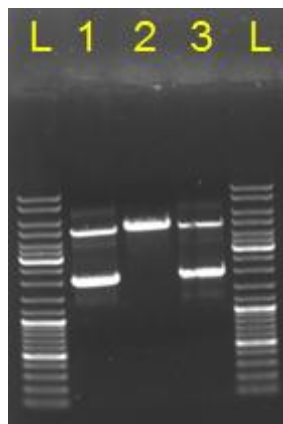


Figure 4 - PCR results. Lane 1 represents CBS8758-2833 (Δhst6 ; *HST6*). Lane 2 represents NRRL-Y-27077 wild type and lane 3 represents the PCR product of the CBS8758-2833 transformed colony.

The PCR product obtained for the NRRL-Y-27077 UF2779 wild type contains only one band, indicating both copies of the *HST6* gene are intact (Figure 4). Lane 1 represents the PCR product obtained for strain CBS8758-2833 where the lower band indicated the one copy of the *HST6* gene that was deleted and the other representing the wild type copy. The profile obtained for the colony obtained after transformation (lane 3) was however similar to that indicating that the second copy of the *HST6* gene was still intact.

5. Conclusion

Results indicate that deletion of *HST6* from the genome of *C. albicans*, using the *SAT1* flipper, was unsuccessful. A previously constructed plasmid (pGem-T Easy Hst6::SAT flipper) was used that contains 500 bp up and downstream of the *HST6* ORF with the *SAT1* flipper in between that. This region was linearized in order to be transformed into the genome of *C. albicans*, for the *SAT1* flipper to delete the *HST6* ORF. Unfortunately, after performing different transformation protocols, none were successful. It was also observed that after transformation, the strains used seem to acquire increased resistance to the antibiotic used for selection. Higher concentration was therefore used to ensure that no growth on the negative control plates was obtained. However, when the concentration was changed to a higher value, no colonies were observed.

While using the *SAT1* flipper method for gene deletion, an article was published by Vyas and co-workers (2015) reporting on the development of a CRISPR-CAS9 gene editing system for efficient gene deletions in *C. albicans*. Due to the problems encountered using the SAT flipper system it was decided to use a CRISPR directed approach to delete *HST6* from the genome of *C. albicans*.

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Chapter 3

Implementing a CRISPR-Cas9 gene editing system in *Candida albicans*

1. Abstract

Candida albicans is a diploid yeast that causes several infections in immune compromised hosts, which may even lead to mortality. Due to its diploidy, gene deletion can be a difficult process, as in some cases two copies of the gene needs to be removed for a phenotype to be observed. We managed to optimise the gene deletion process using the clustered regularly interspaced short palindromic repeat (CRISPR) Cas9 system and implemented it in *C. albicans*, which allows the deletion of several genes in the same genetic background. To validate the CRISPR-Cas9 system the *ADE2* gene was targeted and a double deletion mutant was constructed. When both copies of the *ADE2* gene was removed, the phenotype change from white to red, which can easily be observed. This chapter will discuss the establishment of a CRISPR-Cas9 system for successful gene editing in *C. albicans*.

2. Introduction

Candida albicans is a harmless commensal organism with a diploid genome (Kabir *et al.*, 2012). Due to this diploidy, genetic manipulation is more difficult, as two rounds of excision are required to create double deletion mutants (Jones *et al.*, 2004; Homann *et al.*, 2009). The clustered regulatory interspersed short palindromic repeat (CRISPR)/CRISPR-associated protein (Cas) system (Vyas *et al.*, 2015) was recently described for *Candida albicans*. The CRISPR-Cas9 method for gene disruption relies upon the use of a Cas9 endonuclease and a synthetic guide RNA (sgRNA). The synthetic guide RNA introduces the Cas9 nuclease to create a strand break in the genome, complementary to the sgRNA sequence (Vyas *et al.*, 2015). Regions in the genome that hybridizes to the 20-base pair protospacer from the sgRNA, when it is

followed by the protospacer adjacent motif (NGG), are the targets for cleaving by the Cas9 nuclease.

The first CRISPR-Cas gene editing system for *Candida albicans* was developed by Vyas *et al.* (2015). They constructed a system that consists of a codon optimised Cas9 for *C. albicans* (CaCas9), as the leucine codon (CUG) is translated as serine in this yeast. This ensures compatibility with all CTG clade species. The Cas9 was linked to sequences that encode a FLAG tag and the SV40 nuclear localization signal at the 3' end. The Cas9 was placed under the *ENO1* promoter for expression and the sgRNA, which directs Cas9 for cutting, was placed under the RNA polymerase III promoter SNR52 for expression.

For confirmation of cleaving, *ADE2* was targeted, as a red phenotype indicated that both alleles of the gene are non-functional where the wild type strain or single deletion mutants grow as white colonies (Vyas *et al.*, 2015). As *C. albicans* is diploid, both alleles of most genes need to be non-functional for a different phenotype to be observed. Two systems were created, the Duet system and the Solo system. In the Duet system, two plasmids need to be transformed sequentially; one that contains the CaCas9 that is expressed via the *ENO1* promoter and the second plasmid that contains the sgRNA against *ADE2*, which is targeted to the RP10 locus. The Solo system makes use of one plasmid where the sgRNA and CaCas9 is fused and integrated at the *ENO1* locus and is also double as effective as the Duet system when creating transformants.

When using the Duet system, the antibiotic Nourseothricin (Nat) selects for the integration of the CaCas9 containing plasmid at the *ENO1* locus (Vyas *et al.*, 2015). This marker can be used again for selection, by induction of the flippase gene and the subsequent removal of the *NatR* gene. The Solo system selects for the introduction of the sgRNA using the Nat resistance marker and a variant of the Solo plasmid was constructed that contains a recyclable Nat cassette which allows for other loci to be targeted when additional guide sequences are introduced.

Unfortunately, the above-mentioned system was not available to the wider scientific community. The establishment of an efficient gene editing system based on the CRISPR-CAS9 technology was therefore the aim of this part of the study. For validation of such a system the *ADE2* gene was targeted. Deletion of both copies of

the gene will result in a phenotypic change (from white to red) which will allow an easy observable phenotype to validate the efficacy of the developed CRISP-CAS9 gene editing system.

3. Materials and Methods

3.1 Strains used in this study

Table 1. *Candida albicans* strains used in this study

Strain	Strain number	Genotype
<i>Candida albicans</i>	NRRL-Y-27077	Wild Type
<i>Candida albicans</i>	SC 5314	Wild Type

3.2 Primers used in this study

Table 2. Primers used in this study

Primer name	Primer sequence (5'-3')
HygroB(ORF)-1F	ATGAAAAAGCCTGAACTCACCGCGACG
HygroB(ORF)-1R	AAAAGTACTCTATTCTTTGCCCTCGGACGAG
CaCas9-1F	TTGCCAGAAAAAAGGATTGGG
CaCas9-1R	GATTGATGAATTAAGGTGGCGTC
Ca-ENO1-1F	CTTGTGGGTTTAACATCATTTGTATC
Ca-ENO1-1R	ACGCTAAGGAAAAGAGCCAACA
sgRNA-1F	CGGTGTCTCTGCCAGAAAAAA
sgRNA-1R	TAGATGACGAGGCATTTGGCTA
Ca-25SrRNA-F1	CATAATGATCTTAAGTCGCCCGTCT
Ca-25SrRNA-R1	CGCTAAGGAAAAGAGCCAACA
Ca-25SrRNA-F2-PstI	CCTGCAGCGAAAGGGAATCCGGTTAAA
Ca-25SrRNA-R2-PstI	CCTGCAGAACCTTTCCCACTTCAGTCTT
CaMALprom-CaCas9	CAGTGGTTTAACTAATAATCCAAACGATGGATAA AAAGTATAGTATTG
CaCas9-CaACT1 term	CAGATTTCCAGAATTTCACTCTTATCAAACCTTT CTCTTTTTCTTAG

SATF2_intron	AAGAAGTTGCTGCTTTAGTTAT
SATR2_intron	CACCGTCCATTTTGAATGATTA
SAT flipper 4F	TAAGAGTGAAATTCTGGAAATCTGGAAATC
SAT flipper 4R	GATTATTAGTTAAACCACTGCAAAAAGTTGG
Ca_25SrRNA-F1	CATAATGATCTTAAGTCGCCCGTCT
Ca_25SrRNA-R1	TAGATGACGAGGCATTTGGCTA
Ade2-1F	GATAGCAAACTGTTGGTATTTTAG
Ade2-1R+overlap	AATTTACAATCGAATCATAGTTTG
Ade2-1R+overlap	AATTTACAATCGAATCATAGTTTG
Ade2-2F+overlap	TTGACTGAGATGAACCAATATAT
Ade2-2R	GATTCACCGAAAGTATTATCAACTA

3.3 DNA gel electrophoresis

For visualisation of deoxyribonucleic acid (DNA), gel electrophoresis (0.8% agarose) was performed at 90 volt (V) for 30 min to visualise DNA using a on a Gel Doc™ XR+ (Bio-Rad). The gel was prepared by boiling agarose along with TAE electrophoresis buffer (50x stock: 242 g.l⁻¹ Tris Base, 57.1 ml.l⁻¹ glacial acetic acid, 37.2 g.l⁻¹ Na₂EDTA.2H₂O) and addition of ethidium bromide (5 mg.ml⁻¹) after cooling. The gel was allowed to solidify in a casting tray, along with a comb to create wells. After solidification the comb was removed and the samples (5 µl DNA: 1 µl 6x DNA Loading Dye (Thermo Scientific)) were loaded along with a molecular marker (O'GeneRuler DNA Ladder, Fermentas). This is a standard protocol and was performed throughout the study in the case of visualising DNA.

3.4 Hygromycin B minimum inhibitory concentration (MIC)

The MIC of Hygromycin B were determined using YPD plates (10 g.l⁻¹ Yeast Extract Powder, 20 g.l⁻¹ Peptone Powder, 20 g.l⁻¹ Glucose) supplemented with Hygromycin B at 100 µg.ml⁻¹, 200 µg.ml⁻¹, 400 µg.ml⁻¹ and 600 µg.ml⁻¹ in order to determine concentration that would inhibit the growth of the wild type *C. albicans* strains.

3.5 Transformation

For the initial part of the study, a number of transformation protocols were tested to select the most optimal protocol for transformation of *Candida albicans* using plasmid pKM180. This plasmid is a wide range yeast plasmid that contains 18S rDNA from *Kluyveromyces marxianus* and a Hygromycin resistance gene, which was optimized for *C. albicans*. For integration, the plasmid is linearized with *NotI* (NEB®) prior to transformation resulting in integration at the 18S rRNA loci. For the latter part of the study, only the transformation protocol supplied by Nguyen *et al.* (2017) was used.

3.5.1 Yeast Transformation using Bicine (Klebe *et al.*, 1983)

Preparation of yeast competent cells:

Cells were inoculated in 5 ml YPD broth and incubated with shaking at 37°C overnight. A volume of 5 ml cell culture was inoculated into 100 ml YPD broth and grown to an OD₆₀₀ of 0.6-0.8. Cells were harvested by centrifugation at 1000 x g for 5 min at room temperature (RT) and the supernatant was discarded. Cells were washed once with 50 ml of Solution 1 (1 M sorbitol, 10 mM Bicine-NaOH (pH 8.35), 3% ethylene glycol, 5% (v/v) DMSO) and resuspended in 2 ml of Solution 1. A volume of 200 µl of the cell suspension was aliquoted into 1.5 ml centrifuge tubes and immediately used for transformation or was frozen at -20°C for 4-8 hours and transferred to -80°C.

Transformation:

For fresh cells, 15 µl digested pKM180 (0.1 – 5 µg) and 5 µl carrier DNA (10 µg.µl⁻¹) (denatured at 99°C for 10 min) were added to 200 µl cell suspension and incubated for 5 min at 37°C. The same protocol was used for frozen cells, except that during the incubation step the suspension was mixed every 10 to 15 seconds until completely thawed. For all cells this was followed by the addition of 1.4 ml Solution 2 (40% Polyethylene glycol (PEG) 1000, 200 mM Bicine (pH 8)) and mixed using a vortex for 1 min. Cells were incubated without shaking for 1 h at 30°C, harvested by centrifugation at 5000 x g for 5 seconds at RT and the supernatant was discarded. A volume of 1 ml of Solution 3 (0.15 M NaCl, 10 mM Bicine (pH 8)) was added and the cells carefully resuspended by mixing using a pipette. Cells were harvested by centrifugation at 5000 x g for 5 seconds at RT, the supernatant discarded, and the

cells carefully resuspended in 200 μ l Solution 3. Cells were the plated out on YPD plates containing 600 μ g.ml⁻¹ Hygromycin B and incubated at 37°C for 2 – 3 days.

3.5.2 One Step Yeast Transformation (Chen *et al.*, 1992)

Cells were inoculated into 5 ml YPD broth and grown overnight. An aliquot of 0.8 ml of the culture was centrifuged at 16000 x *g* for 4 seconds at room temperature (RT). The supernatant was discarded using aspiration and 15 μ l (50 ng – 1 μ g) pKM180 (digested with *NotI*) was added as well as 5 μ l (10 μ g. μ l⁻¹) Carrier DNA (SS (Salmon Sperm) (denatured at 99°C for 10 min)). The pellet was loosened with a pipette and resuspended in 100 μ l pre-warmed (45°C) One Step Buffer (0.5 ml 2 M LiOAc, 4 ml 50% PEG and 0.5 ml 1 M dithiothreitol (DTT)) and incubated at 42°C for 30 min. Cells were harvested by centrifugation at 16000 x *g* for 8 second at RT. The supernatant was removed, and cells were resuspended and washed in 0.3 ml YPD broth. A volume of 0.3 ml sorbitol (1 M) was added and cells were resuspended and plated out onto pre-warmed YPD plates containing 600 μ g.ml⁻¹ Hygromycin B.

3.5.3 Transformation using electroporation

Two different electroporation protocols were used to transform *C. albicans* (NRRL-Y-27077 and SC 5314 with pKM180 (digested with *NotI*).

According to the protocol by Wickes and Edman (1994), cells were inoculated and grown overnight in 30 ml YPD broth at 30°C with shaking. An aliquot of 1 ml cells was transferred to 50 ml fresh YPD broth and incubated at 30°C with shaking until an OD₆₀₀ of 1 was obtained. A volume of 30 ml of the culture was centrifuged at 3000*g* for 5 min at 4°C to harvest cells and the supernatant was discarded. Cells were washed twice with chilled ddH₂O (3000 x *g* for 5 min at 4°C) and resuspended in 50 ml cold elution buffer (10 mM Tris-HCl, pH 7.5, 1 mM MgCl₂, 270 mM sucrose (9.24 g/100 ml)) with 200 μ l 1 M DTT. Cells were incubated on ice for 5 – 15 min, harvested by centrifugation at 3000 x *g* for 5 min at 4°C and washed in 30 ml elution buffer without DTT. All but approximately 1 ml of the supernatant was decanted, and cells were resuspended and transferred to a microcentrifuge tube. The cells were harvested by centrifugation at 5000 rpm for 5 min at 4°C, washed (dH₂O) and all but approximately 0.1 ml of supernatant was decanted. Cells were resuspended and 15 μ l pKM180 (1-5 μ g), as

well as 5 μ l SS (10 μ g/ μ l, denatured at 99°C for 10 min), was added. The cell suspension was transferred to cuvettes kept on ice. Electroporation was performed using the following settings: 0.5 kV & 25 μ F. Two hundred microlitres YPD broth was added to the cuvette and cells were transferred to a microcentrifuge tube for incubation at 30°C for 4 hours stationary. *C. albicans* cells were plated out on YPD plates containing 600 mg.l⁻¹ Hygromycin B and incubated for 2 – 3 days at 37°C.

According to the protocol by Kohler *et al.* (1997), cells were grown overnight in 30 ml YPD broth at 30°C with shaking. Five hundred microlitres of cells were inoculated in 50 ml YPD broth and incubated at 30°C with shaking to an OD₆₀₀ of 1.6 – 2.2. Cells were harvested by centrifugation at 3300 x *g* for 5 min at RT and resuspended in 8 ml sterile ddH₂O (double distilled) water. A volume of 1 ml 10x TE buffer (100 mM Tris-HCl, 10 mM EDTA, pH 7.5) as well as 1 ml 1 M LiOAc (Lithium Acetate) (pH 7.5) were added and mixed. The suspension was incubated on a rotary shaker at 150 rpm for 60 min at 30°C. Dithiothreitol (1 M, 250 μ l) was added and incubated on the rotary shaker for 30 min followed by the addition of 40 ml cold sterile ddH₂O. After mixing, the cells were harvested by centrifugation at 3300 x *g* for 5 min at 4°C. Tubes containing the cells were placed on ice and the supernatant was removed. Cells were resuspended in 25 ml sterile cold ddH₂O and centrifuged at 3300 x *g* for 5 min at 4°C. The supernatant was discarded, and cells were resuspended in 5 ml ice-cold 1 M sorbitol and centrifuged at 3300 x *g* for 5 min at 4°C (all steps were performed on ice from here on). The supernatant was discarded, and cells were resuspended in 50 μ l ice-cold 1 M sorbitol. Cell suspension was divided into 40 μ l aliquots and the microcentrifuge tubes were kept on ice. A volume of 5 μ l DNA (pkM180) was added and mixed with electrocompetent cells. Cells were transferred to precooled cuvettes on ice. Electroporation was carried out at 1.8 kV and cuvettes were placed back on ice. A volume of 1 ml sterile 1 M sorbitol was added, mixed with the cells and transferred to a microcentrifuge tube. Cells were centrifuged for 5 min at 450 x *g* at 4°C and resuspended in 1 ml YPD broth. Cells were incubated at 37°C for 4 hours with shaking. Cells were centrifuged for 5 min at 450 x *g* at 4°C and resuspended in 100 μ l 1 M sorbitol and plated out on YPD plates containing 600 mg.l⁻¹ Hygromycin B and incubated for 2 – 3 days at 37°C.

3.5.4 Transformation using Lithium Acetate (LiOAc) (Gietz *et al.*, 1992)

Preparation of yeast competent cells:

Cells were cultivated on YPD medium overnight at 37°C. Colonies were picked from the plate and inoculated in 5 ml YPD broth and incubated overnight with shaking at 37°C. A 50 µl aliquot of culture was inoculated into 5 ml YPD broth and incubated with shaking at 37°C to an OD₆₀₀ of 0.5 – 1.0. Cells were harvested by centrifugation for 2 min at 3000 x g at RT and the supernatant discarded. Cells were washed with 1 ml sterile water and transferred to a microcentrifuge tube and was pelleted at 16000 x g for 10 seconds at RT. Cells were resuspended in 1 ml 1x TE/LiOAc (prepared from 10x TE and 10x LiOAc stock: 1 ml 10x TE stock, 1 ml 10x LiOAc stock, 8 ml sterile water. 10x TE = 100 mM Tris-HCl, 10 mM EDTA, pH 7.5. 10x LiOAc stock = 1 M LiOAc, pH 7.5 with diluted acetic acid). The suspension was kept on ice until transformation or stored at 4°C and used within two weeks.

Transformation:

A volume of 5 µl Carrier DNA (SS - denatured at 99°C for 10 min), 5 µl pKM180 and 100 µl competent cells were mixed by repeated pipetting. A volume of 300 µl pre-warmed (42°C) PEG 40% (2.4 ml 50% PEG 3350, 300 µl 10x TE, 300 µl 10x LiOAc) was added to the solution and mixed carefully by repeated pipetting. This was incubated overnight at 30°C stationary and heat shocked for 15 min at 44°C. Cells were centrifuged for 1 min at 2000 x g at RT and the supernatant was discarded. Cells were washed with 1 ml 1 M sorbitol and resuspended in 100 µl YPD broth. This was plated out on YPD plates containing 800 mg.l⁻¹ Hygromycin B.

3.5.5 Nguyen *et al.* (2017) *Candida albicans* CRISPR Transformation Protocol

A loop full of *C. albicans* SC 5314 cells were inoculated into 5 ml YPD broth and incubated overnight at 30°C with shaking. Fifty microliters were transferred to 5 ml YPD broth and grown to an OD₆₀₀ of 0.5-0.8. Cells were centrifuged for 5 min at 4000 rpm, the supernatant was discarded and cells were washed two times in ddH₂O. Cells were resuspended in 100 µl ddH₂O. Ten microliters carrier DNA (10 mg.ml⁻¹ denatured salmon sperm DNA (denatured at 99°C for 10 min)), 5 µl linearized pMiniT::rRNA (*Pst*I)::pSFS2_SAT::CaCas9 and 50 µl cell suspension were mixed together. One

millilitre fresh PLATE mix (875 μ l 50% PEG 3350, 100 μ l 10x TE (100 mM Tris, pH 7.4, 10 mM EDTA pH 8) and 25 μ l 1 M LiOAc, pH 7) were added and gently mixed by inversion. This suspension was incubated overnight at 30°C stationary.

Cells were heat shocked for 15 min at 44°C, centrifuged at 5000 rpm for 2 min and the supernatant was discarded completely. Cells were resuspended and washed twice with 1 ml YPD broth and 1 ml YPD broth was added to the cell suspension (2 ml total). Cells were incubated for four hours at 30°C with shaking and were centrifuged for 2 min at 5000 rpm. All but 300 μ l of supernatant was discarded and the pellet was resuspended in the YPD broth left. This was plated out on YPD plates containing either 100 or 300 μ g.ml⁻¹ NTC (depending on which plasmid were transformed) and incubated at 30°C for 2-3 days.

3.5.6 Reviving of ordered plasmids and amplification of plasmid DNA

CAS9 was codon optimised for use in *Candida albicans* and ordered from GenScript®. This plasmid, CaCAS9::pESC-URA, was amplified in *Escherichia coli* (NEB® 10-beta Competent Cells) using the rubidium chloride transformation method (Hanahan, 1985). Cells were streaked out on LB (5 g/L yeast extract, 10 g/L tryptone, 10 g/L NaCl and 15 g/L agar) plates supplemented with AIX (Ampicillin (30 mg.ml⁻¹ in dH₂O), IPTG (24 mg.ml⁻¹ dH₂O) and X-gal (20 mg.ml⁻¹ N,N-Dimethylformamide)) and incubated at 37°C overnight.

Plasmids pADH99 & pADH100-2 were ordered from Addgene and arrived as transformed *E. coli* cells. Cells were streaked out on LB plates supplemented with AIX and incubated at 37°C overnight.

3.5.7 Genomic DNA extraction, plasmid extraction & gel purification

Genomic DNA extraction was performed using the ZR Fungal/Bacterial DNA MiniPrep kit according to manufacturer's instructions (Zymo Research) or genomic DNA extraction was performed using the Quick-DNA™ Fungal/Bacterial Miniprep Kit (Zymo Research), according to manufacturer's recommendation. Plasmid extraction was performed using the GenElute™ Plasmid Miniprep Kit (Sigma-Aldrich) according to manufacturer's protocol or using the Zyppy™ Plasmid Miniprep Kit (Zymo Research).

Gel extraction/purification was performed using the QIAquick® Gel Extraction Kit to excise the correct band of the PCR product or via the Bioflux DNA/RNA Extraction/Purification kit (BioSpin Gel Extraction Kit).

3.6 Polymerase Chain Reaction

3.6.1 PCR amplification of ENO1, sgRNA and 25S rRNA

Ca-ENO1-1F and Ca-ENO1-1R primers were used to amplify the *ENO1* ORF with an additional 1000 bp upstream and downstream using genomic DNA. The sgRNA (tRNA (Arg) + *SNR52* promoter) region was amplified using primer pair sgRNA-1F and sgRNA-1R using genomic DNA. Ca-25SrRNA-F1 and Ca-25SrRNA-R1 primers were used to amplify the 25SrRNA region of *C. albicans* using genomic DNA. A PCR was performed to amplify the pMiniT::25SrRNA plasmid using Ca-25SrRNA-F2-PstI and Ca-25SrRNA-R2-PstI primers and to insert a *PstI* restriction site

Amplification of DNA sequences was performed by the use of a KOD Hot Start DNA Polymerase (Novagen®) kit. Standard PCR conditions for all primer sets were as follow: One cycle of a 2 min denaturation at 94°C followed by 25 cycles consisting of a 20 second denaturation at 95°C, 10 seconds annealing at 60°C and a 2 min and 30 seconds elongation at 70°C. This was followed by one final elongation cycle for 7 min at 72°C.

3.6.2 PCR amplification of CAS9

A PCR was performed using CaCAS9::pESC-URA plasmid with primers: CaMALprom-CaCas9 and CaCas9-CaACT1 term. Amplification of DNA sequences was performed by the use of a KOD Hot Start DNA Polymerase (Novagen®) kit according to manufacturer's instructions. PCR conditions were as follow: One cycle of a 2 min denaturation at 94°C followed by 25 cycles consisting of a 20 second denaturation at 95°C, 10 seconds annealing at 60°C and a 2 min and 30 seconds elongation at 70°C. This was followed by one final elongation cycle for 7 min at 72°C.

3.6.3 PCR amplification of the SAT1 Flipper system

A PCR was performed using pSFS2_SAT in pBluescript with primers: SAT flipper 4F and SAT flipper 4R. Amplification of DNA sequences was performed by the use of a KOD Hot Start DNA Polymerase (Novagen®) kit according to manufacturer's instructions. PCR conditions were as follow: One cycle of a 2 min denaturation at 94°C followed by 25 cycles consisting of a 20 second denaturation at 95°C, 10 seconds annealing at 60°C and a 2 min and 30 seconds elongation at 70°C. This was followed by one final elongation cycle for 7 min at 72°C.

3.6.4 PCR amplification of pMiniT::rRNA (*Pst*I)::pSFS2_SAT::CaCas9 (Neb Builder)

A PCR was performed using pMiniT::rRNA (*Pst*I)::pSFS2_SAT::CaCas9 (Neb Builder) with primers: Ca_25SrRNA-F1 and Ca_25SrRNA-R1. Amplification of DNA sequences was performed by the use of a KOD Hot Start DNA Polymerase (Novagen®) kit according to manufacturer's instructions. PCR conditions were as follow: One cycle of a 2 min denaturation at 94°C followed by 25 cycles consisting of a 20 second denaturation at 95°C, 10 seconds annealing at 60°C and a 2 min and 30 seconds elongation at 70°C. This was followed by one final elongation cycle for 7 min at 72°C.

3.6.5 ADE2 donor DNA construction

Fragment A – A PCR was performed using *C. albicans* genomic DNA with primers: Ade2-1F and Ade2-1F+overlap.

Fragment B – A PCR was performed using *C. albicans* genomic DNA with primers: Ade2-2F+overlap and Ade2-2R.

Fragment C – A PCR was performed using Fragment A and B with primers: Ade2-1F and Ade2-2R

Amplification of DNA sequences was performed by the use of a KAPA Taq PCR Kit v5.13 (KAPABIOSYSTEMS). PCR conditions were as follow: One cycle of a 5 min denaturation at 96°C followed by 25 cycles consisting of a 30 second denaturation at

96°C, 30 seconds annealing at 55°C and a 1 min elongation at 72°C. This was followed by one final elongation cycle for 7 min at 72°C.

3.7 Confirmation of DNA integration

For colony PCR: Cells were picked up with a yellow pipette tip and resuspended in 25 µl ddH₂O and boiled by 94°C for 10 min, prior to PCR.

3.7.1 pKM180

To confirm integration of pKM180 a PCR was performed using primers: HygroB(ORF)-1F & HygroB(ORF)-1R. Amplification of DNA sequences was performed by the use of a KOD Hot Start DNA Polymerase (Novagen®) kit according to manufacturer's instructions. PCR conditions were as follow: One cycle of a 2 min denaturation at 94°C followed by 25 cycles consisting of a 20 second denaturation at 95°C, 10 seconds annealing at 60°C and a 2 min and 30 seconds elongation at 70°C. This was followed by one final elongation cycle for 7 min at 72°C.

3.7.2 CAS9

CAS9 integration was confirmed with a PCR reaction using primers: CaCas9-1F and CaCas9-1R. Amplification of DNA sequences was performed by the use of a KAPA Taq PCR Kit v5.13 (KAPABIOSYSTEMS) according to manufacturer's instructions. PCR conditions were as follow: One cycle of a 5 min denaturation at 96°C followed by 25 cycles consisting of a 30 second denaturation at 96°C, 30 seconds annealing at 55°C and a 1 min elongation at 72°C. This was followed by one final elongation cycle for 7 min at 72°C.

3.7.3 ADE2

A PCR was performed using primers: Ade2-1F and Ade2-2R. Amplification of DNA sequences was performed by the use of a KAPA Taq PCR Kit v5.13 (KAPABIOSYSTEMS) according to manufacturer's instructions. PCR conditions were as follow: One cycle of a 5 min denaturation at 96°C followed by 25 cycles consisting of a 30 second denaturation at 96°C, 30 seconds annealing at 55°C and a 1 min elongation at 72°C. This was followed by one final elongation cycle for 7 min at 72°C.

A PCR was performed using primers: Ade2-1F & Ade2-2R. Amplification of DNA sequences was performed by the use of a Expand Long Template PCR System Kit according to manufacturer's instructions. PCR conditions were as follow: One cycle of a 2 min denaturation at 94°C followed by 30 cycles consisting of a 10 second denaturation at 94°C, 30 seconds annealing at 55°C and a 2 min elongation at 68°C. This was followed by one final elongation cycle for 4 min at 68°C.

A PCR was performed using primers: Ade2-1F & Ade2-2R. Amplification of DNA sequences was performed by the use of a KAPA HiFi HotStart ReadyMix PCR Kit. PCR conditions were as follow: One cycle of a 3 min denaturation at 95°C followed by 25 cycles consisting of a 20 second denaturation at 98°C, 15 seconds annealing at 60°C and a 3 min elongation at 72°C. This was followed by one final elongation cycle for 4 min at 72°C.

3.8 Cloning

3.8.1 Cloning of *ENO1*, sgRNA and 25S rRNA

For *ENO1*, sgRNA and 25S rRNA, PCR products were cloned into pMiniT (NEB®) plasmid vector according to manufacturer's protocol and the cloned product was extracted for screening (Sambrook *et al.*, 1989).

3.8.2 Cloning of pMiniT::25SrRNA (*Pst*I)

pMiniT::25SrRNA (*Pst*I) (PCR product) was digested with *Dpn*I and a gel excision on the 4108 bp band was performed. This was digested using *Pst*I and ligated overnight using T4 ligase. Five microliters ligation reaction was used to transform competent *E. coli* cells (NEB® 10-beta Competent Cells) using the rubidium chloride transformation method and was plated on AIX plates. Plasmids were extracted. Double digestion using *Pst*I and *Bgl*II was performed and the 4041 bp band was purified from the gel.

3.8.3 Cloning of pSFS2_SAT (w/o FLP recomb)::CaCas9

The NEBuilder HiFi DNA Assembly Reaction Protocol was performed using the linear pSFS2_SAT (w/o FLP recomb) and CaCas9 gene to create a circular plasmid: pSFS2_SAT (w/o FLP recomb)::CaCas9. This was used to transform *E. coli* cells (NEB® 10-beta Competent Cells) using the rubidium chloride transformation protocol.

Plasmid DNA was extracted and a double RE digest was performed using *Bam*HI and *Pst*II and the 7003 bp band was purified from the gel. Digestion using *Sa*II was also performed.

3.8.4 Cloning of pMiniT::rRNA (*Pst*II)::pSFS2_SAT::CaCas9 (Neb Builder)

Gel purified pMiniT::rRNA (*Pst*II) (4041 bp) and pSFS2_SAT::CaCas9 (Neb Builder) (7003 bp) was ligated according to manufacturer's recommendation (Thermo Scientific T4 DNA Ligase, 5 u/μl) and was transformed using the rubidium chloride transformation protocol. Plasmid DNA was extracted.

3.9 Protein Expression

3.9.1 SDS-PAGE

A loop full of cells were picked up and inoculated into 5 ml YPM (Yeast Peptone Maltose – 10 g Yeast Extract Powder, 20 g Peptone Powder, 20 g Glucose per 1L dH₂O) with 5 μl NTC to a final concentration of 100 μg.ml⁻¹. Cells were incubated with shaking at 37°C overnight and 1 ml of cell suspension was transferred to a microcentrifuge tube. Cells were pelleted, and the supernatant was discarded. Five hundred microliters 50 mM Tris, 0.1% SDS was added along with a PCR tube's volume of glass beads. Cells were shaken 4 times with a Bead Bug at 400 x10 for 60 seconds. Cells were kept on ice and 50 μl was transferred to a new microcentrifuge tube and 50 μl Laemmli Buffer (950 μl Laemmli Buffer with 50 μl β-mercaptoethanol) was added. This was boiled at 96°C for 5 min and 20 μl of this solution was loaded to the SDS-PAGE gel which was prepared according to manufacturer's protocol (TGX™ FastCast™ Acrylamide Kit, 10%, 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 Destaining Method (Fairbanks *et al.*, 1971).

3.9.2 Western Blot analysis

The same procedure was followed with the SDS-PAGE gel, except that the gel wasn't stained. After the gel was run for approximately 1.5 h, the gel, as well as membranes and filter papers, were incubated in Towbin Buffer for approximately 10 min on a Rocker 35 EZ shaker. The sandwich was packed and was run for 30 min at 25 V and

1 A. The membrane was transferred to wash buffer (50 µl Tween20 + 100 ml PBS) for 3 min and the wash buffer was discarded. Blocking buffer (100 µl Tween20 + 10 g milk powder + 200 ml PBS) was added to the membrane and was put on the Rocker 35 EZ for 1 hour with movement. Blocking buffer was discarded and 1 µl antibodies (Anti-CRISPR-Cas9 antibody [7A9-3A3] (HRP) ab202580) + 5 ml blocking buffer was added for 1 h and put on the Rocker 35 EZ to gently mix the antibodies. The solution was discarded, and the membrane was washed 6 times in 15 ml wash buffer for 5 min each, on the Rocker 35 EZ. Three point five millilitres Clarity Western ECI Substrate – Luminol Enhancer Solution (Bio-Rad) and 3.5 ml Clarity Western ECI Substrate – Peroxide Solution was added for 5 min. The solution was discarded, and the membrane was covered in glad wrap. The image was viewed on a Gel Doc™ XR+ (Bio-Rad).

4. Results and discussions

In the case of the following gel electrophoresis images, the letter “L” represents the O’GeneRuler DNA Ladder (Fermentas).

4.1 Testing with pKM180

The pKM180 plasmid is a wide range expression plasmid and was used in this study as a method for screening for the best transformation protocol to use in *Candida albicans*. The plasmid, pKM180 (Figure 1), was digested using *NotI* (Fermentas) to create a linear fragment (Figure 2).

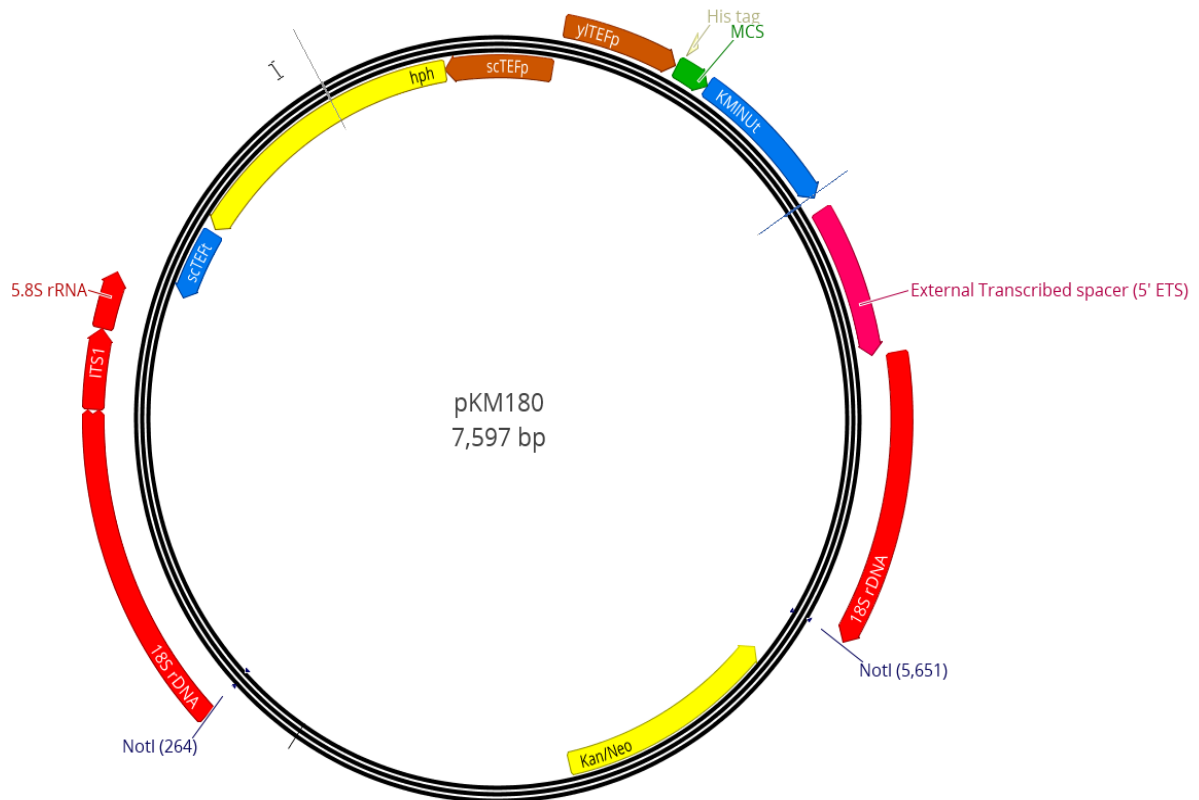


Figure 1 – pKM180 plasmid indicating the important regions as well as the *NotI* restriction sites that were used for linearization of this plasmid.

RE (Restriction enzyme) digest using *NotI* (NEB®) was performed and the correct profile of two bands with approximate sizes of 5400 and 2200 bp was obtained (Figure 2). This digested plasmid was used to transform *C. albicans*.

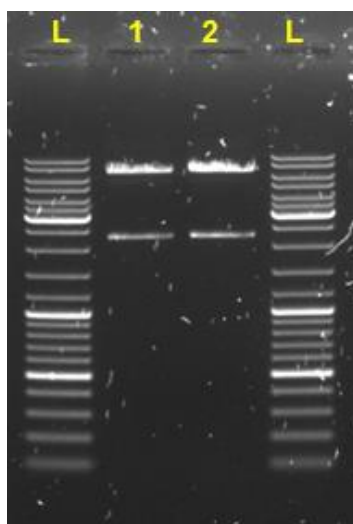


Figure 2 - pKM180 digested using *NotI*. The correct profile was observed after digestion with *NotI*.

4.2 Hygromycin B minimum inhibitory concentration (MIC)

It was observed that for different strains of *C. albicans*, different concentrations of Hygromycin B must be used. NRRL-Y-27077 is more sensitive to the antibiotic than SC 5314 and showed inhibition to Hygromycin B at a concentration of 400 $\mu\text{l.ml}^{-1}$ where SC 5314 showed inhibition at 600 $\mu\text{l.ml}^{-1}$. According to literature (Basso *et al.*, 2010), 600 μl Hygromycin B /ml YPD broth are sufficient to grow cells after transformation, but it was observed that for SC 5314 the concentration had to be higher. When performing MICs, a concentration of 600 μl Hygromycin B /ml YPD were sufficient to inhibit growth for this strain, however, following transformation a concentration of up to 900 μl Hygromycin B $\mu\text{g.ml}^{-1}$ YPD was needed for effective selection of transformed cells.

4.3 Transformation of *C. albicans* with pKM180

Several transformation protocols were used to transform digested pKM180 to select the optimal protocol for transformation of *C. albicans*. The Bicine transformation protocol was initially selected seeing that cells can be made competent and stored at -80°C for further use. Following transformation, cells were plated on YPD plates supplemented with 600 $\mu\text{g.ml}^{-1}$ Hygromycin B. It was however observed that SC 5314 contains multiple single colonies on all plates including the negative control, whereas NRRL-Y-27077 only contained colonies on the negative control plate. The protocol

was repeated several times (5x) and in all cases growth was seen on the negative control plates as well. When a MIC was performed with Hygromycin B to confirm the minimum inhibitory concentration, no colonies were observed at a Hygromycin B concentration of 600 $\mu\text{g}\cdot\text{ml}^{-1}$. However, when cells were plated on YPD plates supplemented with 600 $\mu\text{g}\cdot\text{ml}^{-1}$ Hygromycin B, the negative control still contained growth for both *C. albicans* strains. The one step yeast transformation protocol yielded no growth on any plates for *C. albicans*. For the electroporation protocol by Wickes and Edman (1994) it was observed that single colonies grew on all *C. albicans* plates including the negative control. Similar results were obtained for the protocol by Kohler *et al.* (1997) with growth observed on all plates including the negative control.

When using the Lithium Acetate (LiOAc) transformation protocol with *C. albicans* strain SC 5314 all plates contained growth, including the negative control plates. After the above-mentioned transformation protocols were performed a number of times without any success it was proposed that the pKM180 vector is likely not applicable for use in *C. albicans*.

Parallel to the above-mentioned experiments, a CRISPR-CAS9 gene-editing system was partially developed for use in *C. albicans*. This system was *in-silico* designed to consist of two plasmids containing all the elements needed for a CRISPR-CAS9 system.

The basis of this proposed system was as follows; the *S. pyogenes* CAS9 gene was codon optimized for expression in *C. albicans*, a nuclear localization sequence (NLS) added to the 3'-region (to allow import to the nucleus) and the gene was synthesised by and ordered from GeneScript. To reduce the potential risk that constitutive expression of the CAS9 gene can influence pathogenicity, the CAS9 was fused to the *MAL1* promoter of *C. albicans*. For the gRNA expression it was proposed that a gRNA insertion site is designed and linked to the *C. albicans* snRNA promoter. Both the CAS9 as well as the snR52 regions was then to be inserted into the SAT-flipper cassette (Reuss *et al.*, 2004). The SAT flipper contains the streptothricin acetyl transferase (*SAT1*) gene that confers resistance to Nourseothricin, the Flipase recombinase (*FLP*) gene regulated by the inducible *MAL1* promoter. The complete cassette is flanked by the *FLP* recognition target (FRT) sequences and the complete construct integrated at the rRNA locus. Following homologous integration, cells will be

grown on maltose allowing expression of the FLP-recombinase, this induces recombination between the FRT sequences which removes the complete cassette allowing a second round of genetic deletion.

4.4 Plasmid construction (pMiniT::rRNACaCas9)

For the initial part of the development of a CRISPR-CAS9 system, several of the separate components were amplified and cloned into the pMiniT cloning vector (NEB). Section 4.4.1. shows results obtained following PCR amplification of the snR52 and rRNA regions and cloning of each of these PCR products into the pMiniT (NEB®) cloning vector.

4.4.1 PCR amplification, cloning and RE digest of the sgRNA and 25S rRNA

The PCR amplified sgRNA yielded an expected band of 1553 bp after visualisation by gel electrophoresis (Figure 3).

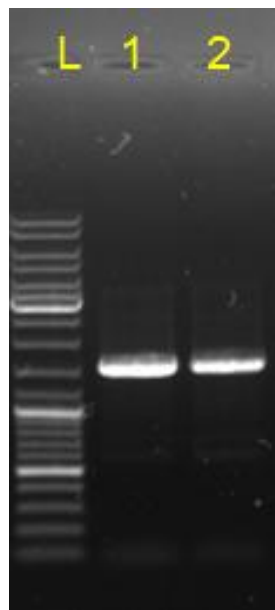


Figure 3 - PCR results. Lanes 1 & 2 shows bands that represent DNA from the amplified sgRNA.

The sgRNA was cloned into the pMiniT (NEB®) plasmid vector and the restriction enzyme digest using *EcoRI* yielded the correct profile of two bands (2486 bp & 1600 bp; lane 9-12) (Figure 4).

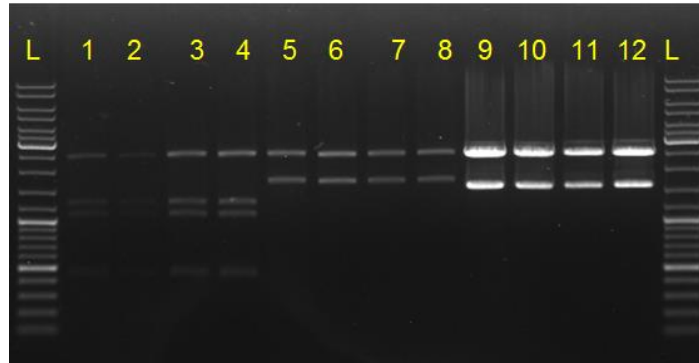


Figure 4 – Restriction enzyme digest results. Lanes 9 - 12 represent the correct profile when digested with *EcoRI*.

The PCR amplified 25S rRNA yielded the expected band of approximately 1650 bp after visualisation by gel electrophoresis (Figure 5).

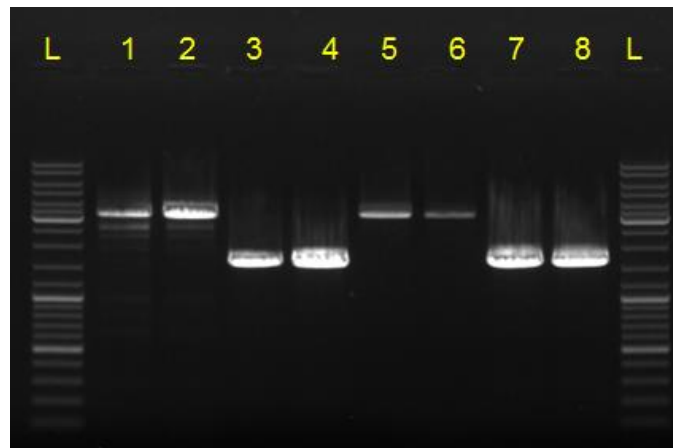


Figure 5 – PCR results. Bands from lanes 3, 4, 7 & 8 represent DNA from the amplified 25S rRNA.

After the 25S rRNA PCR product was cloned into pMiniT (NEB®) plasmid vector, the cloned plasmids were extracted and restriction enzyme digest using *EcoRI* yielded the correct profile of two bands (approximately 2486 bp & 1650 bp) (Figure 6).

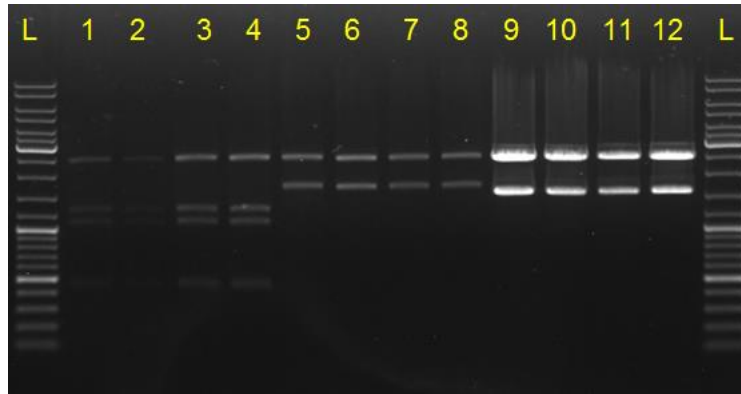


Figure 6 – Restriction enzyme digest results. Lanes 5 - 8 represent the correct profile when digested with *EcoRI*.

To allow subsequent cloning of the CRISPR-CAS9 components, a *PstI* restriction site needed to be incorporated in the central part of the rRNA region. A PCR was performed with primer pair Ca-25SrRNA-R2-*PstI* & Ca-25SrRNA-F2-*PstI*. To both these primers a *PstI* recognition sequence was added and following PCR amplification the PCR product was digested with *PstI* and re-ligated resulting in the addition of a *PstI* restriction sites in the central part of the rRNA region. This resulted in plasmid pMiniT::rRNA (*PstI*) (Figure 7).

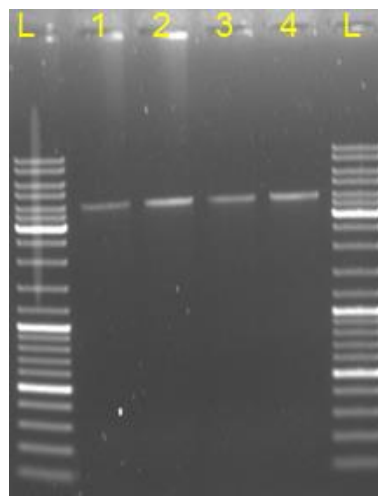


Figure 7 – PCR results. Bands are observed that represent DNA from the amplified pMiniT::25S rRNA plasmid (with *PstI* restriction sites).

The PCR product was digested with *Pst*I, ligated and cloned into *E. coli* competent cells. Plasmid DNA was extracted, and a double digest performed using *Eco*RI and *Pst*I. Insertion of the *Pst*I site was confirmed by a band profile consisting of three bands with sizes of approximately 2500 bp, 900 bp & 700 bp in size (correct profile observed in lane 1 & 2) (Figure 8).

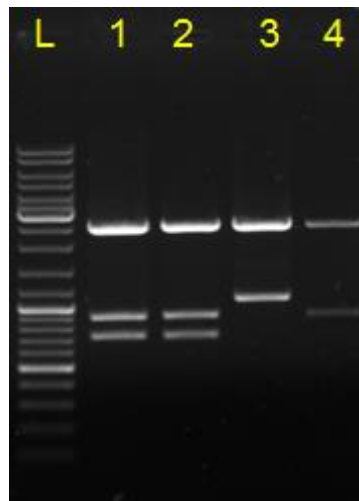


Figure 8 – Restriction enzyme digest results. Lanes 1 – 4 represent pMiniT::25S rRNA (*Pst*I) digested with *Eco*RI & *Pst*I and lanes 1 & 2 shows the correct profile.

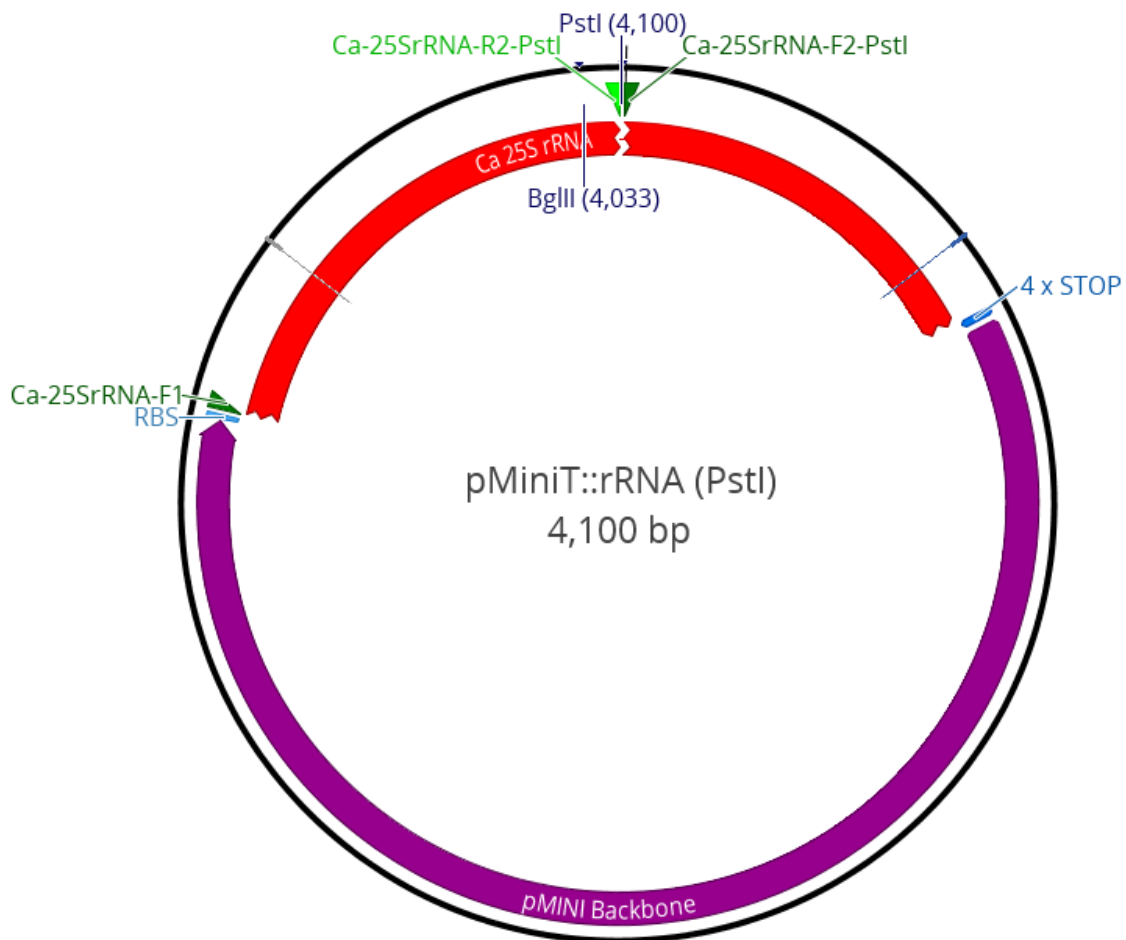


Figure 9 – Map of plasmid pMiniT::25S rRNA (*PstI*). Indicated on the map are the forward (*Ca-25SrRNA-R2-PstI*) and reverse (*Ca-25SrRNA-F2-PstI*) primers used to add a *PstI* site. This site together with the indicated *BglII* restriction sites was used later to clone the *CAS9* region into this plasmid.

4.4.2 Codon optimized *CAS9* amplification and cloning into *SAT1* flipper system

CaCAS9::pESC-URA plasmid containing the *CAS9* gene codon optimised for *C. albicans*, were ordered from Genscript. Restriction enzyme digest using *EcoRI* was performed and the correct profile of two bands was observed (6635 bp & 4237 bp)(Figure 10). RE digest using *HindIII* was performed and the correct profile of three bands was observed (6624 bp, 2463 bp & 1789 bp) (Figure 10).

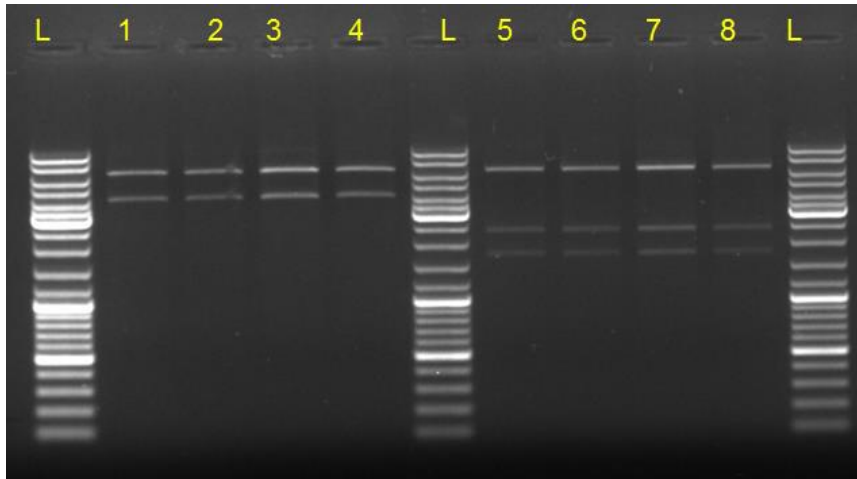


Figure 10 – Restriction enzyme digest results. Lanes 1 – 4 represent CaCAS9::pESC-URA_genscript digested with *EcoRI* and lanes 5 – 8 represent CaCAS9::pESC-URA_genscript digested with *HindIII*.

A PCR was performed to amplify the CaCas9 gene from CaCAS9::pESC-URA plasmid which yielded a fragment of approximately 4247 bp (Figure 11).

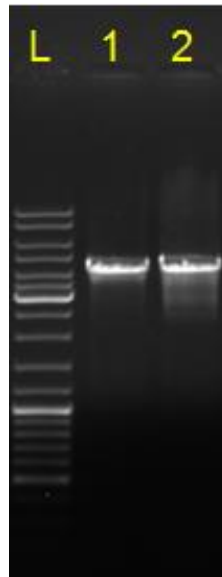


Figure 11 - PCR results. Lanes 1 & 2 represent amplified CaCas9 DNA.

Restriction enzyme digest was performed using *HindIII* and the correct profile of three bands were observed (approximately 2463 bp, 1035 bp & 749 bp) (Figure 12).

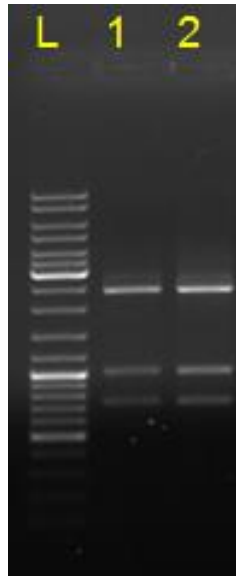


Figure 12 – Restriction enzyme digest results. Lanes 1 & 2 represent digested CaCAS9 using *HindIII*.

To link the *CAS9* gene to the native *C. albicans MAL1* promoter, the *SAT1* flipper plasmid (pSFS2-SAT) was used. In this plasmid the FLP recombinase gene is linked to the *MAL1* promoter. A PCR was performed using primer pair CaCas9-CaACT1 term & CaMALprom-CaCas9 to remove the FLP recombinase, which yielded a band of approximately 5795 bp (Figure 13).

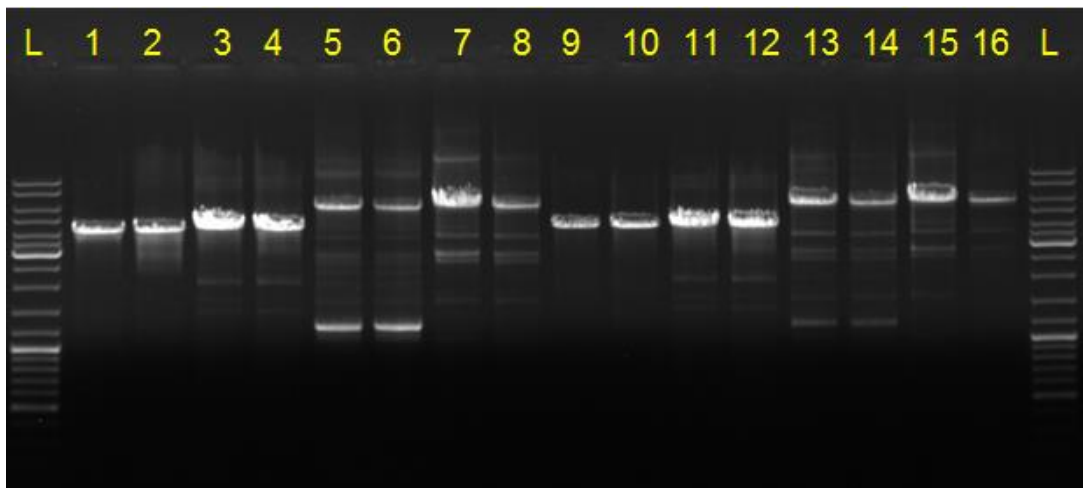


Figure 13 – PCR results. Lanes 7, 8, 15 & 16 represent amplified DNA (using SAT flipper 4F & 4R primers).

Restriction enzyme digest using *BglI* yielded the correct profile of three bands (approximately 2518 bp, 2013 bp & 1270 bp) (Figure 14).

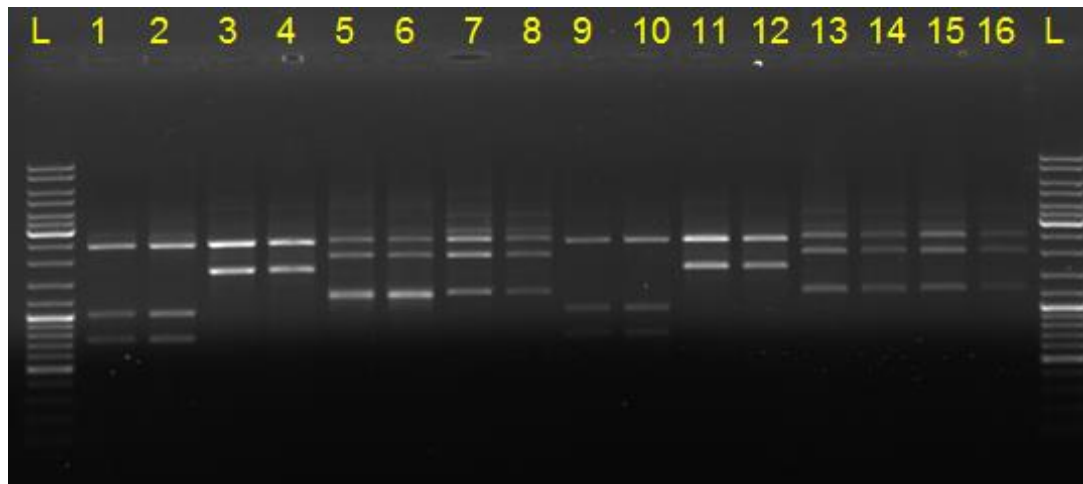


Figure 14 – Restriction enzyme digest results. Lanes 7, 8, 15 & 16 represent digested DNA using *BglI*.

In addition to removing FLP recombinase, the primer pair CaCas9-CaACT1 term & CaMALprom-CaCas9 from plasmid pSFS2-SAT also added an additional 15 bp region that overlaps with the *CAS9* gene. The two fragments (*CAS9* and *SAT1* Flipper plasmid) was fused together using the (NEB®) Builder system from New England Biolabs. The resulting plasmid (pSFS2_SAT (w/o FLP recomb)::CaCas9) therefore contained the *CAS9* gene linked to the *C. albicans MAL1* promoter and *ACT1* terminator regions. Furthermore, it contained the *C. albicans ACT1* promoter – *SAT1* gene-*URA3* terminator. A depiction of the plasmid map of plasmid pSFS2_SAT (w/o FLP recomb)::CaCas9 is supplied below (Figure 15).

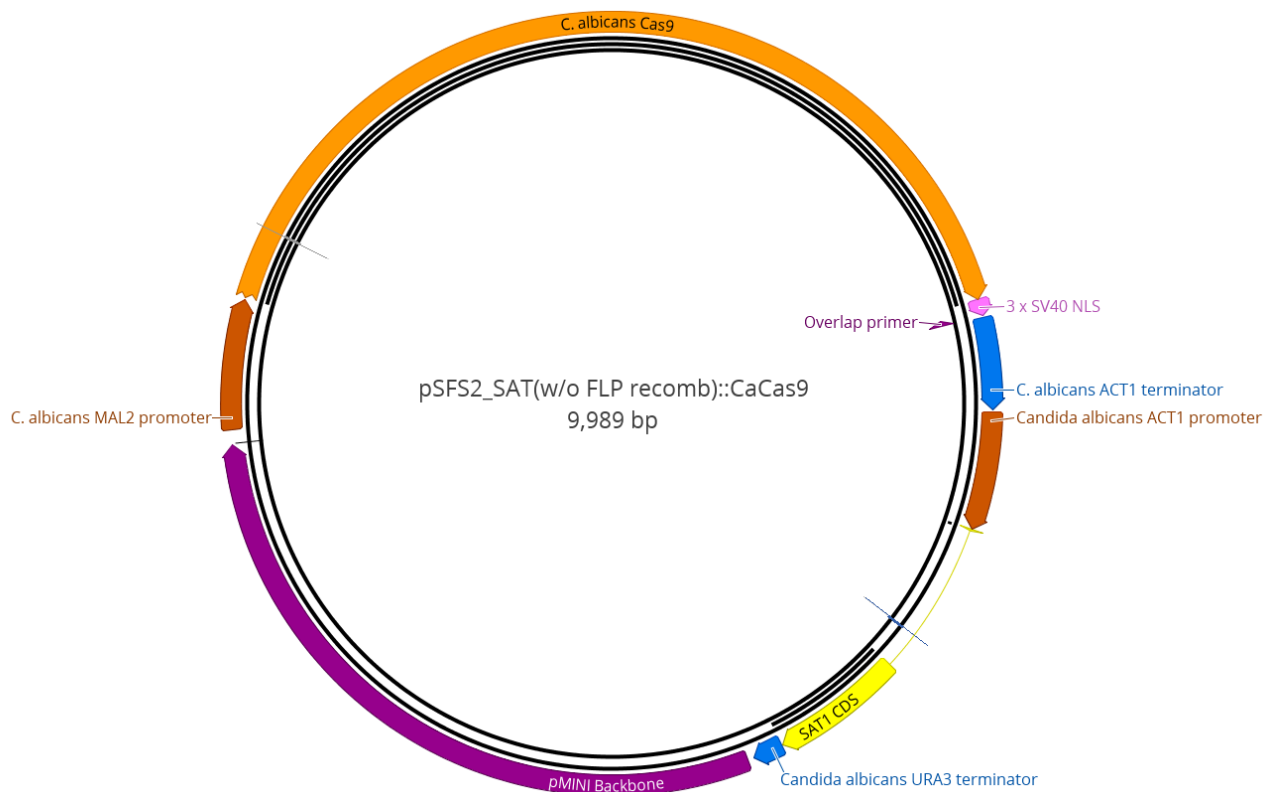


Figure 15 – Plasmid map of pSFS2_SAT (w/o FLP recomb)::CaCas9

Finally ~7000 bp region flanked by the *MAL2* promoter and *URA3* terminator of plasmid pSFS2_SAT (w/o FLP recomb)::CaCas9 (Figure 15) was linked to the *C. albicans* rRNA region (plasmid pMiniT::25S rRNA (*Pst*I)). For this purpose pSFS2_SAT (w/o FLP recomb)::CaCas9 was digested with *Bam*HI and *Pst*I and the ~7000 bp band purified from the gel according to manufacturer’s recommendation (Bioflux DNA/RNA Extraction/Purification (BioSpin Gel Extraction Kit)). pMiniT::25S rRNA (*Pst*I) was digested (linearized) with *Bgl*II and *Pst*I, gel purified and the two fragments were ligated together (note that *Bam*HI and *Bgl*II has compatible sticky ends). Following transformation of the ligation mixture into competent *E. coli* cells, plasmid DNA was extracted, and successful ligation was confirmed through restriction analysis. Restriction digest was performed using *Eco*RI and the correct profile of two bands (Lanes 1 & 2) was observed (bands of approximately 8500 bp & 2500 bp) (Figure 16). Restriction enzyme digest using *Nde*I was performed and the correct profile of three

bands (Lanes 5 & 6) was observed (approximately 7500 bp, 3000 bp & 500 bp) (Figure 16).

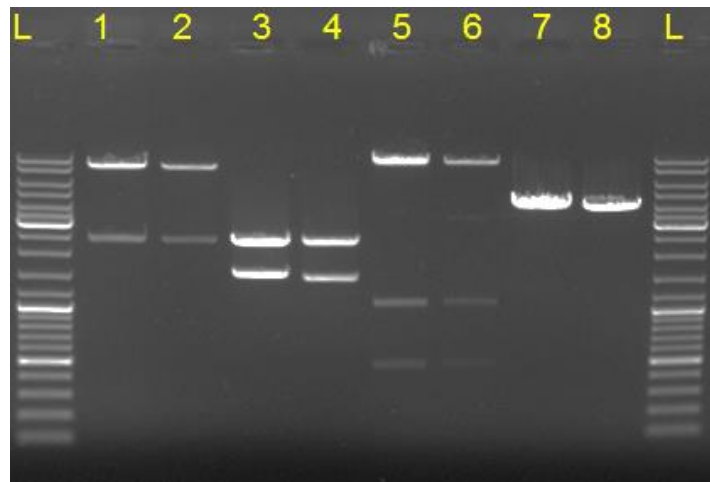


Figure 16 – Restriction enzyme digest results. Lanes 1 & 2 represent the correct profile when pMiniT::rRNA(*Pst*I)::pSFS2_SAT::CaCas9 were digested with *Eco*RI and lanes 5 & 6 the correct profile when digested with *Nde*I.

This resulted in plasmid pMiniT::rRNA (*Pst*I)::pSFS2_SAT::CaCas9 which contained the *C. albicans* codon optimised *CAS9* gene under the control of *MAL2* promoter and *ACT1* terminator. The plasmid also contains the Streptothricin acetyl transferase (*SAT1*) gene that confers resistance to Nourseothricin which is also under the control of the *MAL2* promoter with the *URA3* terminator region fused to the 3'-side of this gene. (Figure 17). These regions were flanked by the *C. albicans* rRNA regions allowing genomic integration at the site of the latter with dominant selection using and Nourseothricin.

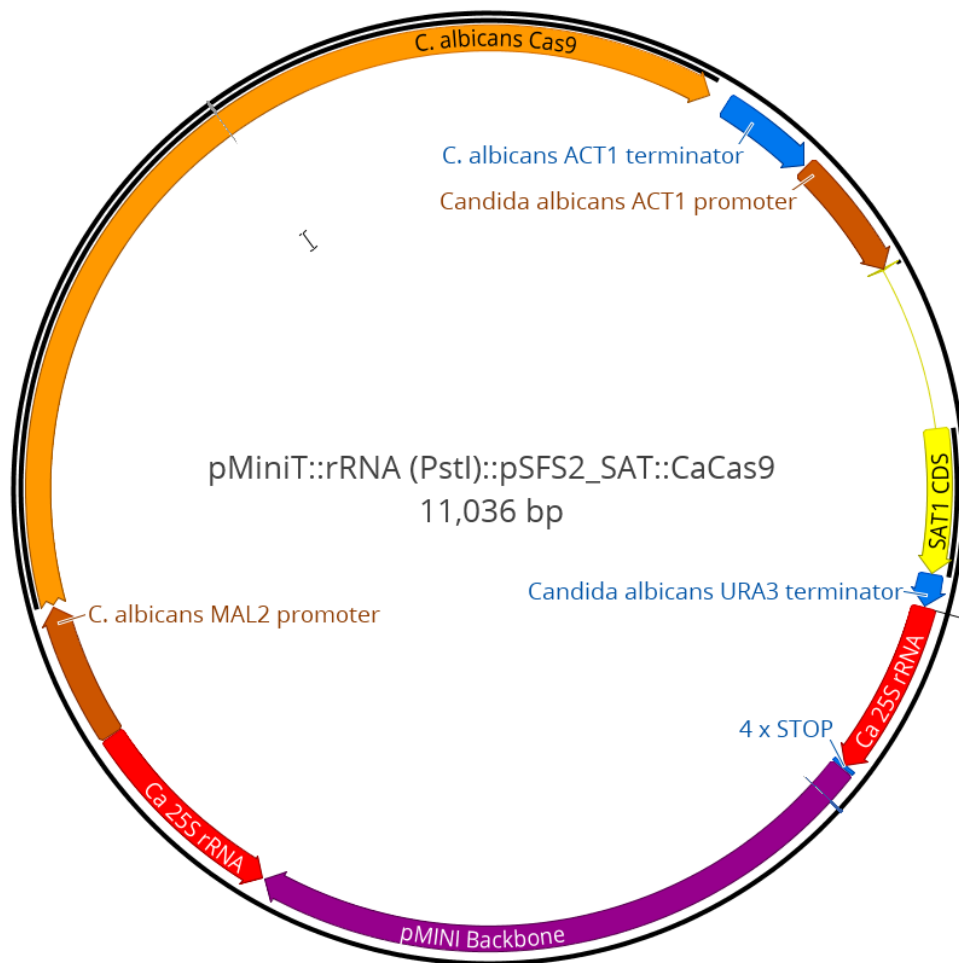


Figure 17 – pMiniT::rRNA (*PstI*)::pSFS2_SAT::CaCas9

4.4.3 CAS9 integration in *C. albicans*.

To integrate the *CAS9* gene in the genome of *C. albicans*, a PCR was performed to create a linear fragment that consists of approximately 8511 bp (Figure 18). For this purpose, primer pair Ca-25SrRNA-F1 & Ca-25SrRNA-R1 was used to amplify the two rRNA regions including the complete region in between.

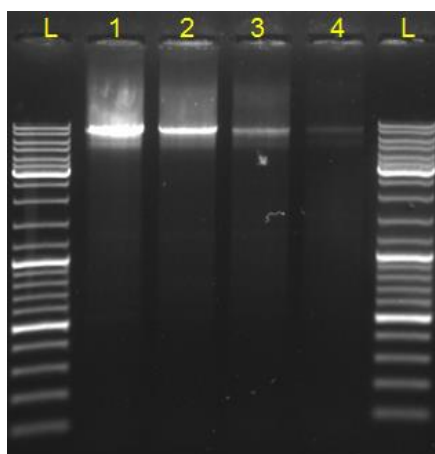


Figure 18 - PCR results. The pMiniT::rRNA(*Pst*I)::pSFS2_SAT::CaCas9 plasmid was linearized using primers Ca_25SrRNA-F1 and Ca_25SrRNA-R1.

Using the PCR product obtained above, *C. albicans* strain SC 5314 was transformed using the Lithium Acetate transformation protocol. Positive transformation was obtained (albeit at very low frequency) and no colonies were observed on the negative control plates. No transformation was obtained for strain NRRL-Y-27077.

Colonies were picked and streaked on YPD plates containing 200 $\mu\text{g}\cdot\text{ml}^{-1}$ NTC and incubated at 30°C overnight. A colony PCR was performed, using two primer pair CaCas9-1F & CaCas9-1R. These two primers amplify a 694 bp region of the *CAS9* gene and positive amplicons therefore indicate successful integration of the complete expression construct. Following PCR one band was seen of approximately 700 bp (Figure 19). These results indicated that the transformation was successful for SC 5314 using the LiOAc method.

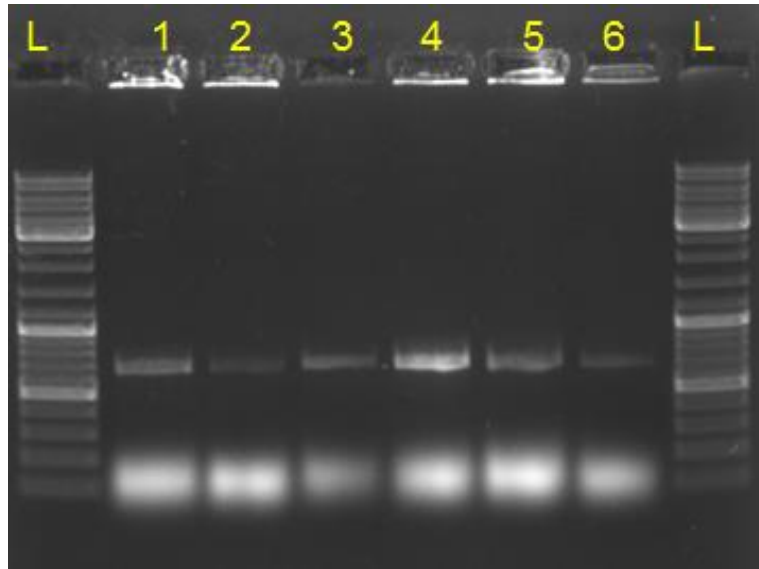


Figure 19 - PCR results. Lanes 1 – 6 represent amplified Cas9 DNA from transformed *C. albicans* SC 5314.

To further optimise the transformation efficacy, the Lithium Acetate method as described by Nguyen *et al.* (2017) was used. Following transformation, colonies were observed on all *C. albicans* plates and no growth was present on the negative control plates. Colonies were picked and streaked on YPD plates containing 200 $\mu\text{g}\cdot\text{ml}^{-1}$ NTC and incubated at 30°C. Colony PCR was performed as described above and one band was seen of approximately 694 bp, which indicated that the transformation was successful (apart from lanes 7 & 9) for both *C. albicans* strains (Figure 20).

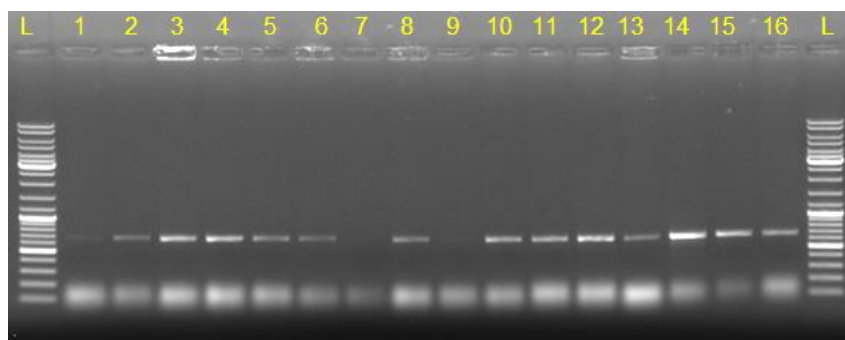


Figure 20 - PCR results. Lanes 1 – 4 represent amplified Cas9 DNA from transformed *C. albicans* NRRL-Y-27077. Lanes 5 – 8 represent amplified Cas9 DNA

from transformed *C. albicans* SC 5314. Lanes 9 – 12 represent amplified Cas9 DNA from transformed *C. albicans* SC 5314 Δ hst6.

4.5 Protein Expression

4.5.1 SDS-PAGE and Western Blot analysis:

To confirm protein expression of the Cas9 gene, an SDS-PAGE gel was run after which a Western Blot analysis was performed. Protein bands were observed after the SDS-PAGE gel was stained (Figure 21).

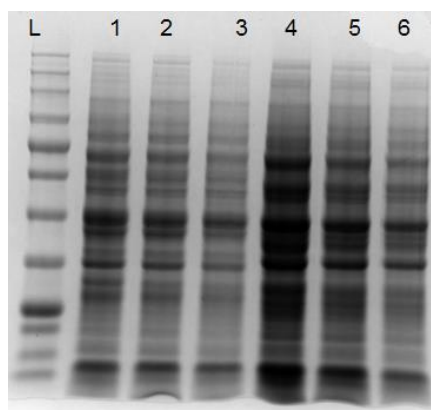


Figure 21 - SDS-PAGE results. Lanes 1 - 6 represent proteins of *C. albicans* SC 5314. Lane 1 represents the negative control SC 5314.

Following western blot analysis, the image was viewed on a ChemiDoc™ XRS+ (Bio-Rad), but no bands were observed after performing this protocol several times (Figure 22). Cells were also collected every hour for 6 hours, but no expression was seen.

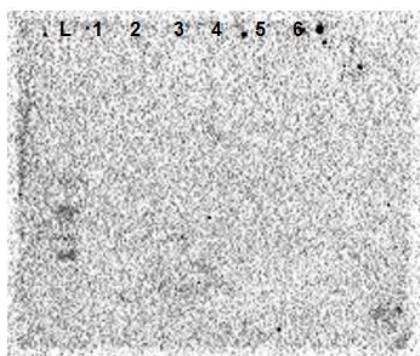


Figure 22 - Western Blot results. Lanes 1 - 6 represent proteins from SC 5314. Lane 1 represents the negative control (untransformed SC 5314).

4.6 Addgene plasmids used in the article by Nguyen *et al.* (2017)

While in the process of construction of the CRISPR system, an article by Nguyen *et al.* (2017) describing the development of an extensive array of plasmids designed for the application of a CRISPR-CAS9 editing system for *C. albicans* was published. All plasmids reported in this article were deposited at Addgene, a non-profit plasmid repository (www.addgene.org/) and these plasmids were subsequently obtained. Because this complete set of CRISPR-CAS9 plasmids was obtained, it was decided to discontinue the development of an in-house system and rather focus on the system as designed by Nguyen *et al.* (2017). A detailed explanation of the principle of this CRISPR-Cas9 system is supplied in chapter 4.

To confirm the application of this system in-house the next section describes the use of this system to delete the *ADE2* gene from *C. albicans*. When the *ADE2* gene is deleted, it results in the accumulation of an intermediate product that gives the yeast cell a pink to red colour on media containing low concentrations of adenine.

Plasmids pADH99 and pADH100-2 were extracted from *E. coli* cells and visualised by gel electrophoresis (Figure 23). The important regions of plasmid pADH99 include the *Candida* codon optimised CAS9, whose expression is regulated by the *ENO1* promoter, the flippase gene linked to the *MAL2* promoter and a 400 bp upstream region of the *HIS3* gene for genome integration of the construct as well as a partial region of the NAT gene that confers nourseothricin resistance. Plasmid pADH100-2 also contain a partial NAT gene that overlaps with a part of the NAT

gene region from pADH99. If these two regions recombine, it will result in the full coding region of the NAT gene. pADH100-2 furthermore contains in tandem the snR52 promoter-*ADE2* CRISPR site-guide RNA scaffold.

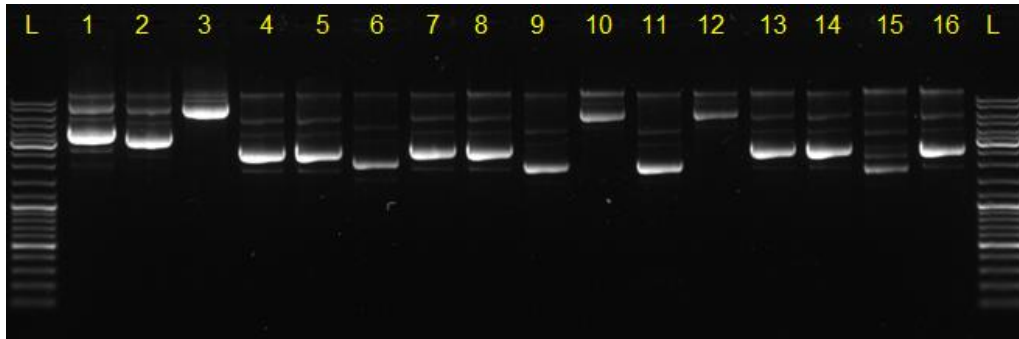


Figure 23 - Plasmid extraction. Lane 3 represents pADH 99, lane 4 pADH 100 and lane 5 pADH 100-2.

pADH99 (Cas9 containing) and pADH100 (guide RNA containing) were analysed using restriction enzymes *Bgl*I and *Mss*I (*Pme*I) (Figure 24). pADH99 digested using *Bgl*I yielded two bands of approximately 9800 bp and 1321 bp and pADH99 digested using *Mss*I (Figure 24) yielded two bands of approximately 8897 bp and 2226 bp. pADH100 digested using *Bgl*I yielded two bands of approximately 2611 bp & 1329 bp and pADH100 digested using *Mss*I yielded two bands of approximately 2226 bp & 1708 bp (Figure 25).

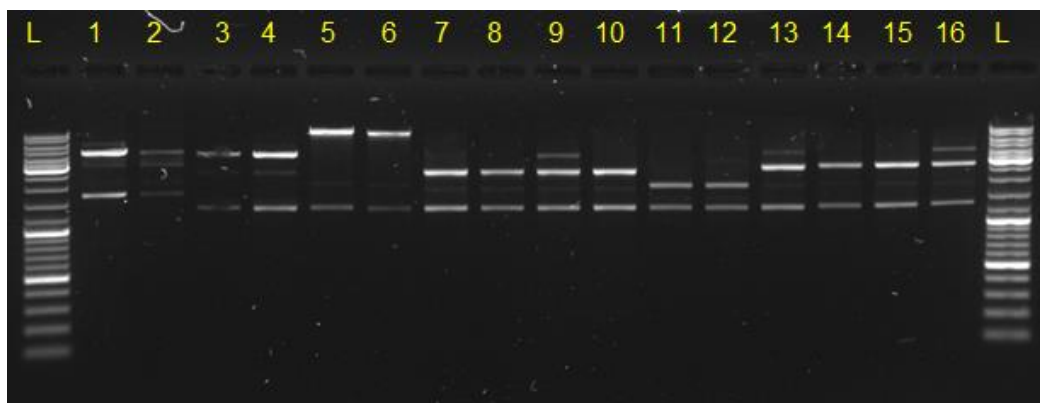


Figure 24 – Restriction enzyme digest results. The following plasmids were digested in duplicate using *Bgl*I, lanes 5 & 6 represent pADH 99, lanes 7 & 8 represent pADH 100 and lanes 9 & 10 represent pADH 100-2.

4.7 Yeast transformations to insert the CAS9 gene and gRNA

Plasmids pADH99 (Cas9 containing) and pADH100-2 (*ADE2* guide RNA containing) were digested using restriction enzyme *MssI*. This separates the CRISPR-Cas9 regions from the plasmid backbone. For pADH99, two bands of approximately 8897 bp and 2226 bp were observed after gel electrophoresis was performed and two bands of approximately 2226 bp and 1710 bp for pADH100-2 were observed (Figure 25).

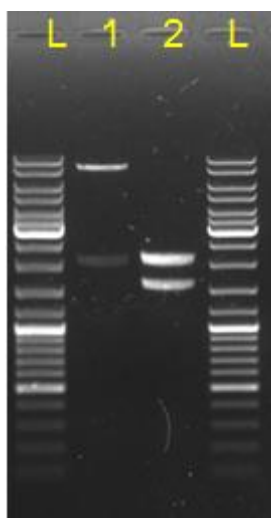


Figure 25 – Restriction enzyme digest results. Lane 1 represents digested pADH99 using *MssI* and lane 2 represents digested pADH100-2 using *MssI*.

These linear fragments were used simultaneously to transform *C. albicans*. It was observed that the plates containing transformed *C. albicans* had growth on all plates except the negative control. Colonies were streaked out on YPD plates containing 300 $\mu\text{g}\cdot\text{ml}^{-1}$ NTC. Six colonies were observed for *C. albicans*.

A colony PCR was performed using two primers that amplifies a region internal to the CAS9. The expected band of approximately 694 bp was obtained, which indicated that the transformation was successful for six SC 5314 colonies (Figure 26).

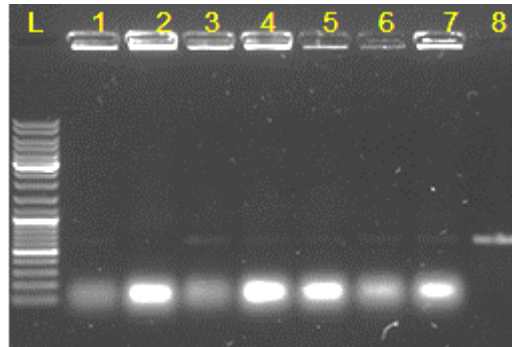


Figure 26 - PCR results. Lanes 1 - 7 represent amplified DNA from transformed *C. albicans* SC 5314 using CaCas9-1F and CaCas9-1R primers. Lane 8 is the positive control which represents amplified DNA from the pADH99 plasmid.

Cells were streaked out on minimal media (YNB+CSM) and incubated for 3 days at 37°C. However, no colour change was observed which indicated that both copies of the *ADE2* gene were still intact.

For efficient gene deletion in *C. albicans*, donor DNA also needs to be added to the transformation mixture. Donor DNA had to be constructed as homology directed repair takes place when the gene is repaired. This will knock out the gene and will prevent the cell from restoring the double stranded break.

4.8 Donor DNA construction

A PCR was performed to yield Fragment A (approximately 182 bp) as well as a second reaction for Fragment B (approximately 276 bp) (Figure 27).

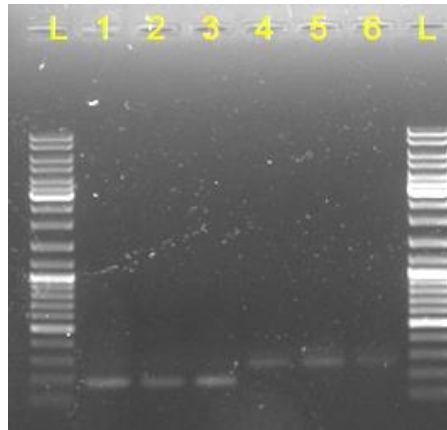


Figure 27 - PCR results. Lanes 1 - 3 represent amplified DNA from *C. albicans* SC 5314 genomic DNA using Ade2-1F and Ade2-1R+overlap primers (Fragment A). Lanes 4 - 6 represent amplified DNA from *C. albicans* SC 5314 genomic DNA using Ade2-2F+overlap and Ade2-2R primers (Fragment B).

Both fragments A and B were used in a PCR reaction using primers Ade2-1F and Ade2-2R, to yield Fragment C (approximately 458 bp) (Figure 28).

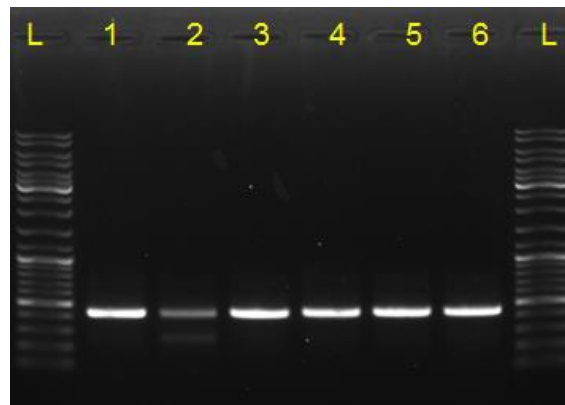


Figure 28 - PCR results. Lanes 1 - 6 represent amplified DNA from Fragment A and B using Ade2-1F and Ade2-2R primers to yield Fragment C.

4.9 Yeast transformations to insert Cas9 gene and gRNA with added donor DNA to delete *ADE2*

Plates with transformed *C. albicans* had growth on all YPD plates containing 200 $\mu\text{g}\cdot\text{ml}^{-1}$ NTC, excluding the negative control. Colonies were streaked out on YPD plates containing 300 $\mu\text{g}\cdot\text{ml}^{-1}$ NTC and 60 colonies were observed after incubation at 37°C. All 60 colonies grew on the higher concentration NTC. After 2 days of incubation 4 out of 60 colonies turned red (pink) (Figure 29). After optimization, 106 colonies were observed, all of the 106 grew on the higher concentration NTC and 25 became red (pink) (Figure 30). Following these results, cells were left overnight on the bench top after the 4 hours outgrowth phase.



Figure 29 - Transformation results. Four out of 60 colonies became red, which indicated that both copies of the *ADE2* gene were deleted.

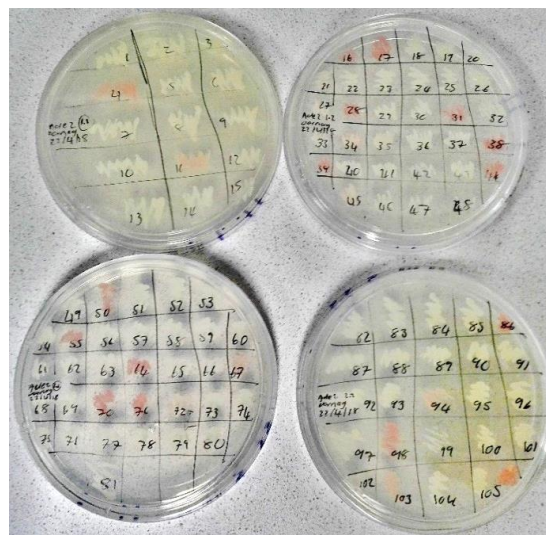


Figure 30 - Transformation results. Twenty-five out of 106 colonies became red after leaving cells on the bench top overnight (after the outgrowth phase).

Colony PCR was performed on 18 of the colonies that turned red. An expected band of approximately 485 bp, indicating that both copies of the gene are deleted, were obtained for three of the colonies (Figure 31; Lane 1, 7 & 17).

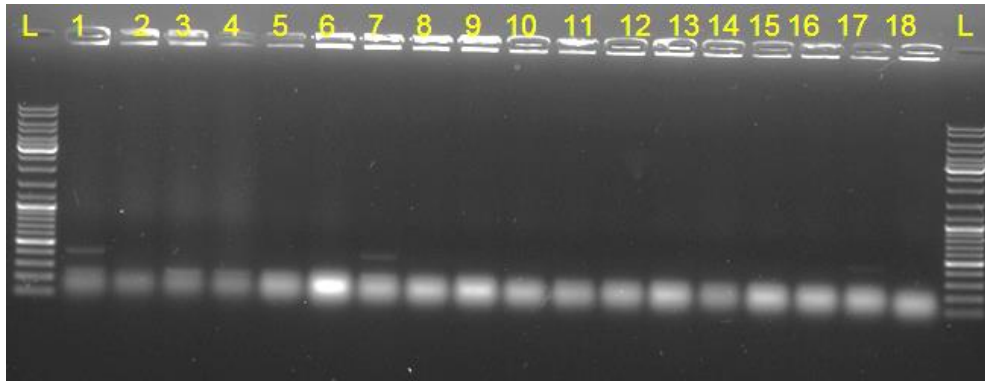


Figure 31 – PCR results. Lanes 1, 7 & 17 represent amplified DNA from *C. albicans* transformed cells.

A genomic DNA extraction was performed on 12 colonies using the ZR Fungal/Bacterial DNA MiniPrep kit according to manufacturer's instructions (Zymo Research). A PCR was performed, and it was observed that 1 out of 12 colonies showed that both copies of the gene were deleted (Figure 32; lane 1). For the wild type, one band of approximately 1868 bp was observed (Figure 32 lane WT).

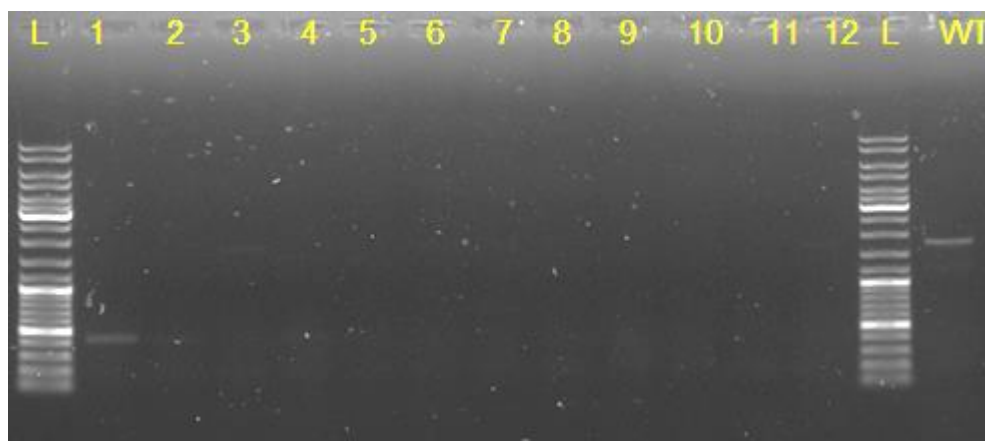


Figure 32 - PCR results. Lanes 1 represents amplified DNA from *C. albicans* transformed cells. The WT showed a band of approximately 1868 bp.

As can be seen from figure 33, 3 out of 6 colonies showed a band of approximately 485 bp which indicated successful removal of the *ADE2* gene. It can also be observed that all 6 colonies were successfully transformed with *CAS9*.

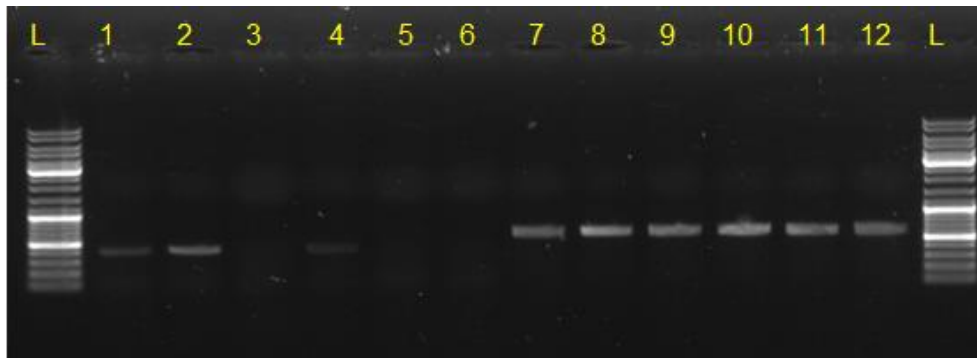


Figure 33 - PCR results. Lanes 1 - 6 represent amplified DNA from *C. albicans* transformed cells using primers Ade2-1F & Ade2-2R. Lanes 7 - 12 represent amplified DNA from *C. albicans* transformed cells using primers CaCas9-1F & CaCas9-1R.

A second genomic DNA extraction was performed on 8 colonies, 4 red, 2 pink and 2 white (Figure 34).

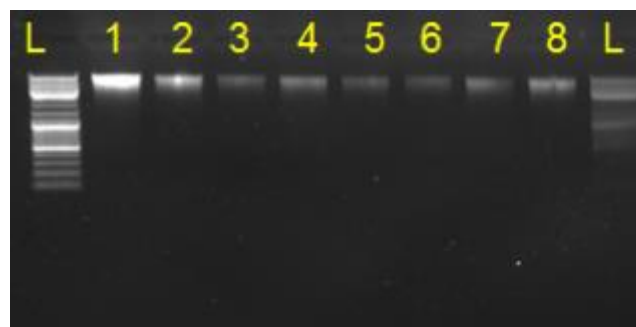


Figure 34 - Genomic DNA extraction results. Lanes 1 – 2 represent DNA from white colonies, lanes 3 – 6 represent DNA from red colonies and lanes 7 – 8 represent DNA from pink colonies.

As can be seen from Figure 35, almost no bands were observed when a colony PCR was performed. Only lane 4 showed a band of approximately 300 bp, which is the incorrect size. The bands representing WT DNA yielded a band of approximately 1868 bp, as did the bands that represent DNA from white colonies. Lanes 12 and 14

represent DNA from red colonies and as expected, only one band of approximately 458 bp was observed, which indicated that the gene was removed. Lane 13 yielded inconsistent results. One of the pink colonies (lane 17) yielded bands of approximately 1868 bp & 458 bp, which indicated that only one allele of the gene was removed. The other pink colony contains both copies of the gene as one band of approximately 1868 bp was observed (lane 16). Lane 15 yielded a band of approximately 800 bp, therefore samples were sequenced.

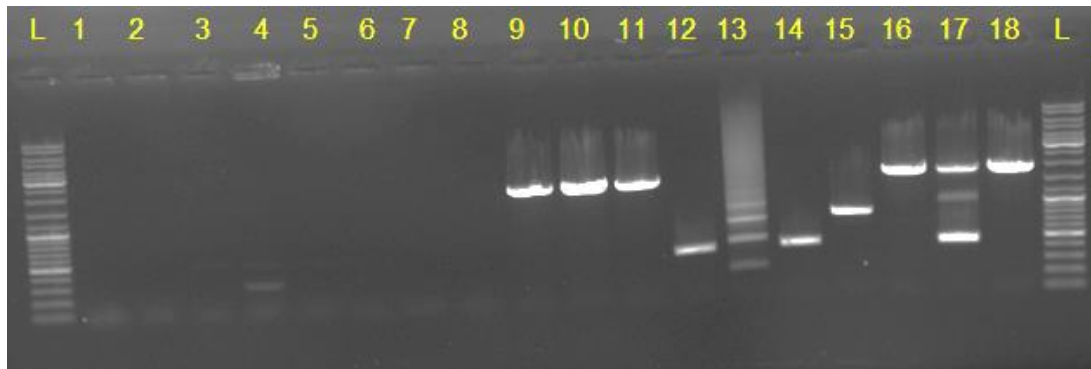


Figure 35 - PCR results. Lanes 1 – 18 represent amplified DNA using KAPA HiFi HotStart ReadyMix PCR Kit. Lanes 1 – 8 represent amplified DNA from whole cells. Lanes 9 & 18 represent amplified DNA from genomic DNA of *C. albicans* (WT). Lanes 10 – 17 represent amplified DNA from genomic DNA. The order is as follow: DNA from 2 white colonies followed by 4 red colonies and two pink colonies.

As can be seen from Figure 36, almost no bands were observed when a colony PCR was performed. Only lane 6 showed bands of approximately 800 bp & 485 bp. The band representing WT DNA (Lane 9) yielded a band of approximately 1868 bp, as did the bands that represent DNA from white colonies (Lanes 10 & 11). Lanes 12 and 14 represent DNA from red colonies and as expected, only one band of approximately 458 bp was observed, which indicated that the gene was removed. Lane 13 yielded inconsistent results. One of the pink colonies (lane 17) yielded bands of approximately 1868 bp, 1000 bp & 458 bp. The other pink colony yielded bands of approximately 1868 bp & 1000 bp. (lane 16). Lane 15 yielded a band of approximately 800 bp, therefore samples were sequenced. Lane 18 (WT) yielded no bands.



Figure 36 - PCR results. Lanes 1 – 18 represent amplified DNA using Expand Long Template PCR System Kit. Lanes 1 – 8 represent amplified DNA from whole cells. Lanes 9 & 18 represent amplified DNA from genomic DNA of *C. albicans* (WT). Lanes 10 – 17 represent amplified DNA from genomic DNA. The order is as follow: DNA from 2 white colonies followed by 4 red colonies and two pink colonies.

The same results were observed and as can be seen from Figure 37, the red colonies lost the WT band of approximately 1868 bp, which indicated that the *ADE2* gene was removed.

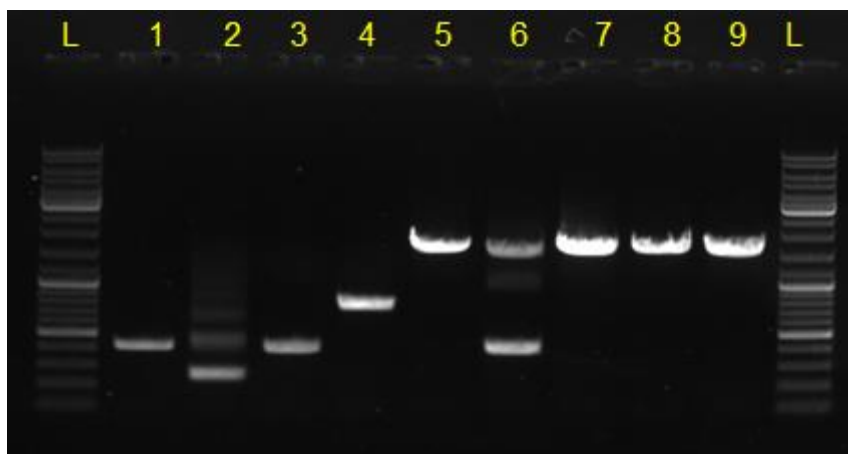


Figure 37 - PCR results. Lanes 1 – 9 represent amplified DNA using KAPA HiFi HotStart ReadyMix PCR Kit. Lanes 1 – 4 represent amplified DNA from red colonies, lanes 5 – 6 represent amplified DNA from pink colonies and lanes 7 – 8 represent amplified DNA from white colonies. Lane 9 represents amplified DNA of *C. albicans* (WT).

As it was observed that the transformation and gene deletion, was successful, the removal of other genes could continue.

5. Conclusion

The initial part of this section described the development of a CRISPR-CAS9 gene editing system for *C. albicans*. Successful integration of the *CAS9* gene into the genome of *C. albicans*, was confirmed using PCR. Unfortunately, no bands were observed when a Western Blot was performed several times to confirm expression of the Cas9 protein (a band of approximately 150 kDa was expected). This result did not conclude that the construct was ineffective, but rather that investigation was needed on the Western Blot process and the antibodies. Note that the integration had to be successful for colonies to be observed as the transformants contains an NTC selection marker. By the time the Cas9 containing plasmid was constructed, an article by Nguyen *et al.* (2017) was published reporting on the availability of a CRISPR-Cas9 system for *C. albicans*. Because this complete set of CRISPR-CAS9 plasmids was obtained from Addgene, it was decided to discontinue the development of an in-house system and rather focus on the system as designed by Nguyen *et al.* (2017).

The two plasmids, pADH99 (Cas9 containing) and pADH100-2 (*ADE2* gRNA containing) for *ADE2* deletion was digested using *MssI* and transformed simultaneously into *C. albicans*. A phenotypic change had to be observed after gene deletion, but colonies stayed white. At this stage, it was realised that for successful deletion of a gene in *C. albicans* the addition of donor DNA is essential. The donor DNA was constructed according to the protocol by Nguyen *et al.* (2017) and transformed along with the digested plasmids. Sixty colonies were observed and after incubation for 24 hours, four out of 60 colonies turned reddish/pink. The process was optimised, and cells were left overnight on the bench top after the outgrowth phase. According to Satomura *et al.* (2017) incubation for 48 hours will best suit this process as Homology Directed Repair (HDR) is induced in the end of the S and G2 phases (HDR is the process in which nicks in the gene is repaired). Twenty-five out of 105 colonies showed a phenotypic change and performing a PCR indicated that the *ADE2* ORF was removed. It was seen that some of the pink cells had only one copy of the gene removed and some red cells had both copies removed where the WT and the white cells had both copies of the gene intact. This confirmed the construction of the

ADE2 deficient colonies and indicated that the process was successful and ready for further use. These results yielded an optimised CRISPR-Cas9 gene editing system in *C. albicans*, which will allow several genes to be removed in the future.

6. References

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Chapter 4

Construction of a *HST6* homozygous deletion mutant using a CRISPR-Cas9 gene editing system

1. Abstract

Candida albicans forms part of the normal microbiota of human beings and when the immune system becomes compromised, can cause several infections. Changes in the environment of the organism must be monitored and acted upon. The ATP-binding cassette transporters, which includes the *HST6* gene, plays a role in monitoring these changes. *HST6* was previously identified to play a role in several stress responses, such as osmotic, cell wall and heat stress. Also, the effect of fluconazole on deletion mutants of *HST6* showed an increased susceptibility to the drug when one copy of the gene was removed (unpublished results). To investigate the effect of fluconazole on a null mutant (where both copies of the gene are removed), a double deletion mutant of the gene had to be constructed. We previously managed to implement a *C. albicans* CRISPR-Cas9 system to create double deletion mutants of *ADE2* and used this system as a basis to construct a *HST6* double deletion mutant in *C. albicans*. We also managed to create a complemented strain where *HST6* is re-introduced into the genome of the mutants. Biofilms were formed with the double deletion mutant, complemented strain and the wild type. Incubation with fluconazole was performed and an XTT assay revealed that biofilm metabolic activity was reduced 55.8% for the wild type, 75.1% for the double deletion mutant and 70.3% for the complemented strain. Results indicated that the double deletion mutant is more susceptible to fluconazole than the wild type, however the complemented strain is also more susceptible. These results allow for future work to investigate the susceptibility of these mutants to fluconazole.

2. Introduction

Candida albicans is an opportunistic fungal pathogen that can grow in the yeast, pseudohyphae and true hyphae morphology (Sudbery *et al.*, 2004). True hyphae contain paralleled side walls without constrictions at septal junctions where pseudohyphae are formed by elongation and septum formation which results in filaments consisting of elongated cells with constrictions at the septa (Odds, 1988), both are referred to as filamentous.

This organism also needs to monitor changes in its environment to adjust to the host's conditions, which is where the ATP binding cassette (ABC) transporters play a role (Klein *et al.*, 2011). *HST6* is an ABC transporter which was previously indicated to have an influence on various stress conditions, including osmotic, heat and heavy metal stress (Motaung, 2015). Also, based on unpublished results (PhD results, Mr. O. Kuloyo) it was observed that deletions of *HST6* resulted in strains being more sensitive to fluconazole, which is an azole antifungal drug used for treatment of candidiasis (Dupont and Drouhet, 1988).

According to results obtained from Chapter 2, it was observed that creating a double deletion mutant of *HST6* in *C. albicans* was unsuccessful when the *SAT1* flipper system was used. It was decided to use the CRISPR-Cas9 system, developed by Nguyen *et al.*, (2017), to create a double deletion mutant of *HST6* in *C. albicans*. This CRISPR-Cas9 system relies upon the Cas9 endonuclease and guide RNA which directs the Cas9 for double stranded cleaving. This system can be used on any nourseothricin (NTC) sensitive strain of *C. albicans* and relies upon the selection of transformants that contain the NTC resistance gene after integration of the plasmid DNA that contains the NAT (nourseothricin) split-marker. This system can be reused when the marker is removed to allow for the add-back of a previously removed gene or for deletion of a second gene. Growing cells on maltose will induce the *MAL2* promoter to activate the *FLP* gene which will allow the *FRT* sequence to recombine and flip out the NTC marker.

Three plasmids are used in this study namely pADH99, pADH110 and pADH147. One contains the Cas9 endonuclease (pADH99) (Figure 1) and the other two (pADH110 and pADH147) (Figure 2 & 3) are used in a stitching protocol to create the insertion of a specific CRISPR target site (*HST6* target site in this case) (Figure 5).

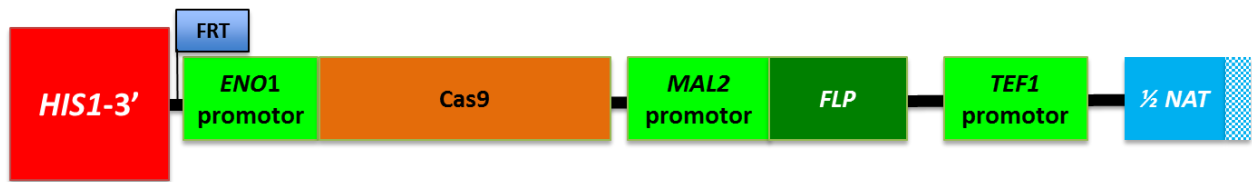


Figure 1 – pADH99. Digested (*MssI*) plasmid containing Cas9 which integrates at the Histidine 1 locus and contains part of the NAT (nourseothricin) split marker.

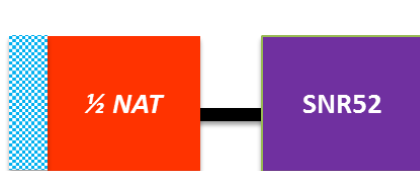


Figure 2 - pADH110. Linear fragment (Fragment A) containing part of the NAT split marker and the SNR52 promoter.

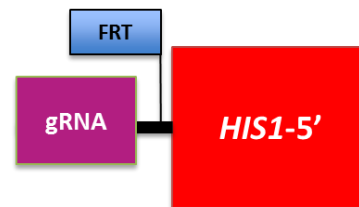


Figure 3 - pADH147. Linear fragment (Fragment B) containing the gRNA region which integrates at the Histidine 1 locus.

To link the two fragments depicted in Figure 2 and 3, an overlap primer is designed containing the desired 20 bp CRISPR target site (Figure 4) flanked by a 20 bp overlap of the snR52 promoter and the gRNA scaffold on the 5' and 3'sides, respectively (Figure 4). A PCR is performed to create one linear fragment (Fragment C) (Figure 5) containing the CRISPR site, the one half of the NAT split marker, the SNR52 promoter, the gRNA and the 5'-side of the *HIS1* integration site.



Figure 4 - Overlap primer. This primer overlaps with the SNR52 promoter of pADH110 and gRNA of pADH147 to insert a *HST6* CRISPR target site for cleavage

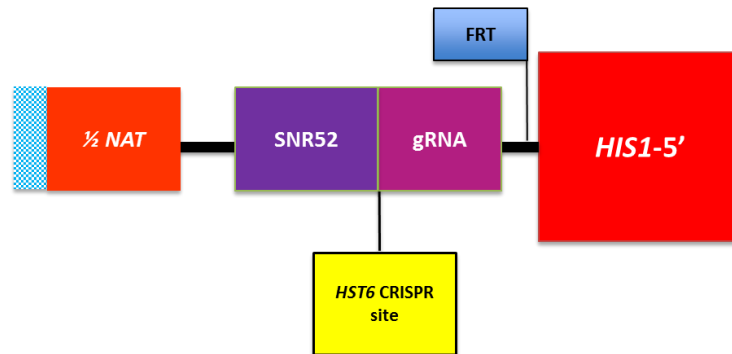


Figure 5 - Fragment C. Linear fragment containing the desired *HST6* CRISPR target site.

Donor DNA should also be constructed by amplifying two fragments (each on either side) of the *HST6* open reading frame and via overlap primers will yield one linear fragment (Figure 6).

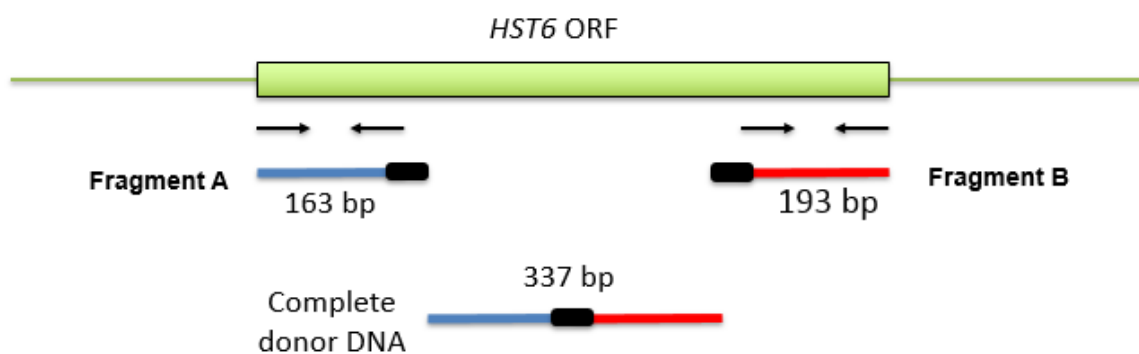


Figure 6 - Donor DNA construction. By amplification of two different fragments of the *HST6* ORF (Fragment A & B), one fragment can be created via overlap PCR (337 bp), which is the donor DNA (Fragment C).

This linear fragment is the donor DNA and must be transformed along with the digested pADH99 and Fragment C to remove the *HST6* gene. Donor DNA are used as a repair template at the target locus in the case of the homology directed repair system.

The aim of this part of the study was therefore to use the CRISPR-Cas9 method for gene deletion in order to elucidate the effect of fluconazole on *HST6* in *C. albicans*.

3. Materials and Methods

3.1 Strains used in this study

Candida albicans strain CBS58758 UF2833 (SC 5314) was used throughout this Study.

3.2 Primers used in this study

Table 1 - Primers used in this study.

Primer name	Primer sequence (5'-3')
HST6_Ca-qF1	TCACCATCAGATTCACCGGCT
Ca_HST6-6R	CGCATTAACTCTTTGTCGTGAGTG
Ca_HS6-6F+overlap	GAAATAATGGAGGTGGACAGTTACAACACTAC
Ca_HST6-6R+overlap	TGTCCACCTCCATTATTTCCCCGTAAAT
Ca_4F + NcoI	CCCATGGGTGGTGGCTTTCCTACGATAA
Ca_HST6-2R	CCGAACCAATCAAATCTCCAGAAGGT
AHO1096-ver2	GACGGCACGGCCACGCGTTTAAAC
AHO1098-ver2	CAAATTA AAAATAGTTTACGCAAGTCTCG

AHO1097	CCCGCCAGGCGCTGGGGTTTAAACACCG
AHO1237	AGGTGATGCTGAAGCTATTGAAG
AHO1236	TAAAGCTGCCACAAGAGGTATTTTC
HST6-CRISPR- 1-oligo*	GTAAACTATTTTTAATTT <u>GTGACTAGTGGTTTCATATGAGGGTTT</u> TAGAGCTAGAAATAG [§]
HST6-CRISPR- 2 oligo ^{‡§}	GTAAACTATTTTTAATTT <u>GGATTGGCCATTGATTCTTGTGGGTTT</u> TAGAGCTAGAAATAG [§]
HST6-CRISPR- 3 oligo*	CGTAAACTATTTTTAATTT <u>GACTCACCTATAAAGCTCAACGTTTT</u> AGAGCTAGAAATAGC [§]
HST6-CRISPR- 4 oligo [‡]	CGTAAACTATTTTTAATTT <u>GCAACAATTAATATCCAACGAGTTTT</u> AGAGCTAGAAATAGC [§]

*Oligonucleotides containing the CRISPR site for use in the *HST6* deletion

[‡]Oligonucleotides containing the CRISPR site for use in the *HST6* add-back

[§]CRISPR site is underlined

3.3 DNA gel electrophoresis

For visualisation of deoxyribonucleic acid (DNA), gel electrophoresis (0.8 % (w/v) agarose) was performed at 90 volt (V) for 30 min to visualise DNA on a Gel Doc™ XR+ (Bio-Rad). The gel was prepared by boiling agarose along with 1x TAE electrophoresis buffer (50x stock: 242 g.l⁻¹ Tris Base, 57.1 ml.l⁻¹ glacial acetic acid, 37.2 g.l⁻¹ Na₂EDTA.2H₂O) and addition of ethidium bromide (5 mg.ml⁻¹) after cooling. The gel was allowed to solidify in a casting tray, along with a comb to create wells. After solidification the comb was removed and the samples (5 µl DNA: 1 µl 6x DNA Loading Dye (Thermo Scientific)) were loaded along with a molecular marker (O'GeneRuler DNA Ladder, Fermentas). This is a standard protocol and was performed throughout the study in the case of visualising DNA.

3.4 *HST6* donor DNA construction

For the construction of *HST6* donor DNA, a PCR was performed using HST6_Ca-qF1 and Ca_HST6-6R+overlap primers with *C. albicans* genomic DNA to yield Fragment A as well as a second reaction using primers Ca_HST6-6F+overlap & Ca_HST6-6R for Fragment B. KAPA Taq PCR Kit v5.13 (KAPABIOSYSTEMS) was used to amplify the DNA sequences. PCR conditions were as follows: One cycle of a 5 min denaturation at 96°C followed by 25 cycles consisting of a 30 sec denaturation at 96°C, 30 sec annealing at 55°C and a 1 min elongation at 72°C. This was followed by one final elongation cycle for 7 min at 72°C. Both fragments A and B were used in a PCR reaction with the same conditions as above, using primers HST6_Ca-qF1 & Ca_HST6-6R, to yield Fragment C which is the donor DNA to be used in the transformation.

For the construction of *HST6* donor DNA for the add back of *HST6*, a PCR was performed using primers Ca_4F + *Nco*I and Ca_HST6-2R with *C. albicans* genomic DNA to yield a fragment of approximately 4 905 bp. KAPA HiFi HotStart PCR Kit (KAPABIOSYSTEMS) was used to amplify the DNA sequence. PCR conditions were as follow: One cycle of a 3 min denaturation at 95°C followed by 25 cycles consisting of a 20 sec denaturation at 98°C, 15 sec annealing at 60°C and a 3 min elongation at 72°C. This was followed by one final elongation cycle for 4 min at 72°C.

3.5 *HST6* CRISPR site insertion using cloning free stitching

Plasmid pADH110 was used to amplify Fragment A with primers AHO1096-ver2 and AHO1098-ver2. KOD Hot Start DNA Polymerase (Novagen®) was used to amplify the DNA sequences with the following conditions: One cycle of a 30 sec denaturation at 94°C, followed by 30 cycles consisting of a sec denaturation at 94°C for 20 sec, 20 sec annealing at 58°C and a 30 sec elongation at 70 °C. This was followed by one final elongation cycle for 7 min at 70°C.

Plasmid pADH147 was used to amplify Fragment B with primers AHO1097 and HST6-CRISPR-*-oligo with * indicating the CRISPR sequence for either the deletion or the add back of *HST6*. KOD Hot Start DNA Polymerase (Novagen®) was used to amplify the DNA sequences with the following conditions: One cycle of a 30 sec denaturation at 94°C, followed by 10 cycles consisting of a sec denaturation at 94°C for 20 sec, 20

sec annealing at 65°C (reducing annealing temperature by 1°C each cycle) and a 20 sec elongation at 70°C. This was followed by 25 cycles consisting of a third denaturation at 94°C for 20 sec, 20 sec annealing at 55°C and a 20 sec elongation at 70°C. This was followed by one final elongation cycle for 5 min at 70°C.

Fragment C was constructed by using KOD Hot Start DNA Polymerase (Novagen®) with the following conditions: One cycle of a 30 sec denaturation at 94°C, followed by 5 cycles consisting of a sec denaturation at 94°C for 20 sec, 20 sec annealing at 58°C and a 40 sec elongation at 70°C. This was held at 20°C for 2 minutes for the addition of primers AHO1237 and AHO1236. This was followed by 30 cycles consisting of a third denaturation at 94°C for 30 sec, 20 sec annealing at 58°C and a 40 sec elongation at 70°C. This was followed by one final elongation cycle for 5 min at 70°C.

3.6 Yeast transformations

A loop full of *Candida albicans* cells were inoculated into 5 ml YPD broth and incubated overnight at 37°C with shaking. A hundred microliters were transferred to 5 ml YPD broth and grown to an OD₆₀₀ of 0.5-0.8 at 30°C with shaking. Cells were centrifuged for 5 min at 4000 rpm, the supernatant was discarded and cells were washed two times in ddH₂O. Cells were resuspended in 100 µl ddH₂O. Ten microliters carrier DNA (10 mg.ml⁻¹ denatured salmon sperm DNA (denatured at 99°C for 10 min)), 10 µl digested (*M*ssI) pADH99, 45 µl Fragment C, 45 µl donor DNA and 50 µl cell suspension were mixed together. One millilitre fresh PLATE mix (875 µl 50% (w/v) PEG 3350, 100 µl 10x TE (100 mM Tris, pH 7.4, 10 mM EDTA pH 8) and 25 µl 1 M LiOAc, pH 7) were added and gently mixed by inversion. This suspension was incubated overnight at 30°C stationary.

Cells were heat shocked for 15 min at 44°C, centrifuged at 5000 rpm for 2 min and the supernatant was discarded completely. Cells were resuspended and washed twice with 1 ml YPD broth and 1 ml YPD broth was added to the cell suspension (2 ml total). Cells were incubated for four hours at 30°C with shaking and were left on the bench to overnight. Cells were centrifuged for 2 min at 5000 rpm. All but 300 µl of supernatant was discarded and the pellet was resuspended in the YPD broth left. This was plated out on YPD plates containing 200 µg.ml⁻¹ NTC and incubated at 30°C for 2-3 days. Resultant colonies were picked up and streaked out on YPD plates containing 300 µg.ml⁻¹ NTC and incubated at 37°C overnight.

For confirmation, a colony PCR was performed for *HST6* transformed colonies. A colony was selected and picked up by touching the colony with the tip of a pipette tip. The cells were resuspended in 25 µl ddH₂O and boiled at 94°C for 10 min. Primers HST6_Ca-qF1 and Ca_HST6-6R were used to amplify a 337 bp region when both copies of the gene are deleted and a band of approximately 3 800 bp when both copies are intact. KAPA Taq PCR Kit v5.13 (KAPABIOSYSTEMS) was used to amplify the DNA sequences. All of the 25 µl boiled cells were used in the PCR reaction, according to manufacturer's recommendation. PCR conditions were as follows: One cycle of a 5 min denaturation at 96°C followed by 25 cycles consisting of a 30 sec denaturation at 96°C, 30 sec annealing at 55°C and a 1 min elongation at 72°C. This was followed by one final elongation cycle for 7 min at 72°C.

3.7 PCR confirmation of *HST6* deletion, add back and Cas9 integration

A genomic DNA extraction was performed on colonies using the ZR Fungal/Bacterial DNA MiniPrep kit according to manufacturer's instructions (Zymo Research). A PCR was performed, using primers HST6_Ca-qF1 and Ca_HST6-6R to confirm *HST6* deletion with KOD Hot Start DNA Polymerase (Novagen®). PCR conditions were as follows: One cycle of a 5 min denaturation at 96°C followed by 25 cycles consisting of a 30 second denaturation at 96°C, 30 seconds annealing at 50°C and a 2 min elongation at 72°C. This was followed by one final elongation cycle for 7 min at 72°C.

When using KAPA HiFi HotStart ReadyMix PCR Kit for *HST6* deletion confirmation, the protocol consisted of: One cycle of a 3 min denaturation at 95°C followed by 25 cycles consisting of a 20 sec denaturation at 98°C, 15 sec annealing at 60°C and a 3 min elongation at 72°C. This was followed by one final elongation cycle for 4 min at 72°C.

When using Expand Long Template PCR System Kit for *HST6* deletion confirmation, the protocol consisted of: One cycle of a 2 min denaturation at 94°C followed by 30 cycles consisting of a 10 second denaturation at 94°C, 30 sec annealing at 55°C and a 3 min and 35 sec elongation at 68°C. This was followed by one final elongation cycle for 4 min at 68°C.

When a colony PCR was performed a colony was selected and picked up by touching the colony with the tip of a pipette tip. The cells were resuspended in 25 µl ddH₂O and

boiled at 94°C for 10 min. Primers HST6_Ca-qF1 & Ca_HST6-6R were used to amplify a 337 bp region when both copies of the gene are deleted and a band of approximately 3 800 bp when both copies are intact.

For confirmation of add back, primers Ca_4F + NcoI and Ca_HST6-2R were used, along with KAPA HiFi HotStart PCR Kit (KAPABIOSYSTEMS) to amplify the DNA sequence. PCR conditions were as follow: One cycle of a 3 min denaturation at 95°C followed by 25 cycles consisting of a 20 sec denaturation at 98°C, 15 sec annealing at 60°C and a 3 min elongation at 72°C. This was followed by one final elongation cycle for 4 min at 72°C.

To amplify the Cas9 gene, Expand Long Template PCR System kit was used, and conditions were as follows: One cycle of a 5 min denaturation at 96°C followed by 25 cycles consisting of a 30 sec denaturation at 96°C, 30 sec annealing at 55°C and a 1 min elongation at 72°C. This was followed by one final elongation cycle for 7 min at 72°C.

3.8 Removal of the NTC marker

In order to add the gene back, the NTC marker had to be removed. This was done by growing cells where *HST6* was deleted in 5 ml YPM (yeast extract-peptone-maltose) media with shaking at 37°C for 24 hrs. Cells were streaked out for single colonies on YPD media and incubated at 37°C for 24 hrs. From that, single colonies were streaked out in duplicate (on YPD plates containing 200 µg.ml⁻¹ NTC and on YPD plates) and incubated at 37°C for 24 hrs. Colonies that showed growth on YPD plates and not on NTC plates were selected and inoculated for transformation as this indicated that the NTC marker was removed successfully.

3.9 Phenotypic analysis

Candida albicans WT (CBS8758 (SC 5314)), *C. albicans* $\Delta\Delta$ hst6 (CBS8758 (SC 5314)) and *C. albicans* $\Delta\Delta$ hst6::*HST6HST6* (CBS8758 (SC 5314)) were streaked out on YPD agar and was incubated overnight at 37°C. A loop full of cells was inoculated into 5 ml YPD broth and cells were incubated with shaking at 37°C. A dilution series was made (10^{-1} , 10^{-2} , 10^{-3} and 10^{-4}) and 10 µl of the diluted cells were pipetted on YPD plates and was incubated at 37°C for 48 h. Phenotypic changes were noted.

3.10 Biofilm formation and fluconazole sensitivity

Candida albicans WT (CBS8758 (SC 5314)), *C. albicans* $\Delta\Delta$ hst6 (CBS8758 (SC 5314)) and *C. albicans* $\Delta\Delta$ hst6::*HST6HST6* (CBS8758 (SC 5314)) were streaked out on YM agar and was incubated for 24 hours at 30°C. A loop full of cells were inoculated into 5 ml YNB broth (6.7 g/L Yeast Nitrogen Base) supplemented with 1% glucose and incubated with shaking at 30°C for 24 h. Cells were harvested at 3000 x g for 5 min and was washed twice with 10 ml of sterile PBS buffer. Cells were resuspended in 5 ml sterile PBS buffer, counted with a haemocytometer and diluted with filter sterilized RPMI-1640 medium to a final concentration of 1×10^6 cells.ml⁻¹. Diluted cells (100 μ l) were added into 96-well microtiter plates (Costar®, USA) and was incubated at 37°C for 90 min to allow adherence of cells. Non-adherent cells were washed twice with sterile PBS buffer and wells drained before proceeding. RPMI media (200 μ l) containing 1 mg.l⁻¹ fluconazole was added to the wells and incubated at 37°C for 48 h to allow biofilm formation. Wells were washed twice with 200 μ l sterile PBS. A XTT (2,3-bis(2-methoxy-4-nitro-5-sulfophenyl)-5[(phenylamino) carbonyl]-2H tetrazolium hydroxide) assay was performed on the biofilms. Four hundred microliters Menadione (1 mM) was added into 5 ml XTT and 50 μ l of the solution was added into each well. The microtiter plate was incubate in the dark for 3 h and the absorbance of each well measured at 492 nm. This experiment was performed in triplicate.

4. Results and Discussions

4.1 Yeast transformations of the CRISPR-Cas9 construct

Using this CRISPR-Cas9 system (Nguyen *et al.*, 2017), Cas9, donor DNA and guide RNA must be transformed simultaneously. Cas9 was obtained by digesting plasmid pADH99 using restriction enzyme *M*ssI. Two bands of approximately 8897 bp and 2226 bp were observed after gel electrophoresis was performed (Figure 7).

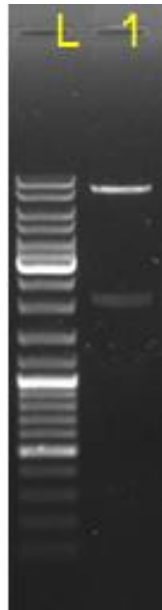


Figure 7 - Restriction enzyme digest results. Lane 1 represents digested pADH99 using *MssI*.

For donor DNA construction, a PCR was performed to yield Fragment A (approximately 154 bp) as well as a second reaction for Fragment B (approximately 183 bp) (Figure 8).

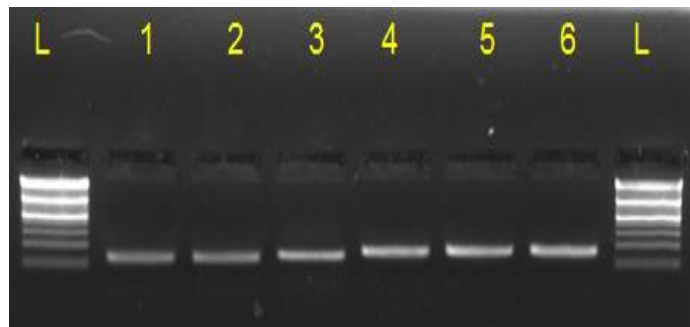


Figure 8 - PCR results. Lanes 1 - 3 represent amplified DNA from *C. albicans* WT cells using primers HST6_Ca-qF1 and Ca_HST6-6R+overlap. Lanes 4 - 6 represent amplified DNA from *C. albicans* WT cells using primers Ca_HST6-6F+overlap & Ca_HST6-6R (O'GeneRuler DNA Ladder, Fermentas was used for a shorter period of time (+/- 5 min).

A PCR was performed using Fragment A and Fragment B in a single PCR reaction to yield Fragment C (donor DNA - approximately 337 bp) (Figure 9).

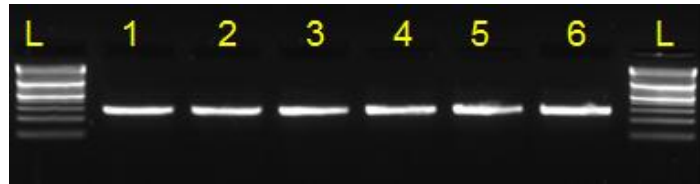


Figure 9 - PCR results. Lanes 1 - 6 represent amplified DNA from Fragment A and Fragment B using HST6_Ca-qF1 and Ca_HST6-6R primers.

For the complete gRNA to be constructed, a cloning free stitching method was performed using plasmids pADH110 and pADH147. For *HST6* CRISPR site 1 insertion using cloning free stitching, a PCR was performed on plasmid pADH110 to yield Fragment A containing part of the NAT split marker and the SNR52 promoter (approximately 1 065 bp) as well as a second reaction using plasmid pADH147 for Fragment B (approximately 663 bp) containing the gRNA region which integrates at the Histidine 1 locus (Figure 10).

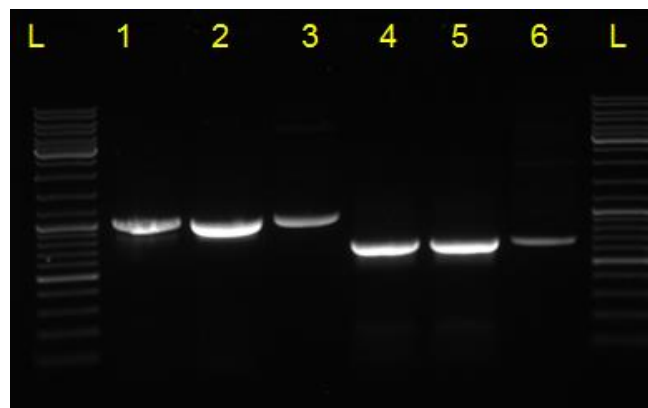


Figure 10 - PCR results. Lanes 1 - 3 represent amplified DNA of Fragment A using primers AHO1096-ver2 & AHO1098-ver2 and lanes 4 - 6 represent amplified DNA of Fragment B using primers AHO1097 & HST6-CRISPR-1-oligo.

A PCR was performed using Fragments A and B with primers AHO1237 & AHO1236 (approximately 1 729 bp) to yield fragment C which is the complete gRNA with the desired *HST6* CRISPR site (Figure 11).

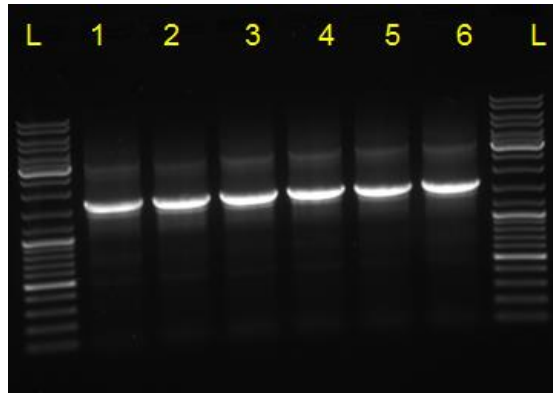


Figure 11 - PCR results. Lanes 1 - 6 represent amplified DNA from Fragment A and Fragment B using AHO1237 & AHO1236 primers.

Cas9, donor DNA and fragment C (gRNA) was transformed simultaneously and it was observed that the plates with transformed *C. albicans* had growth on all YPD plates containing 200 $\mu\text{g}\cdot\text{ml}^{-1}$ NTC, excluding the negative control plates. Colonies (85) were streaked out on YPD plates containing 300 $\mu\text{g}\cdot\text{ml}^{-1}$ NTC and 84 of the colonies showed growth after overnight incubation at 37°C. Six random colonies were selected and genomic DNA was extracted (Figure 12).

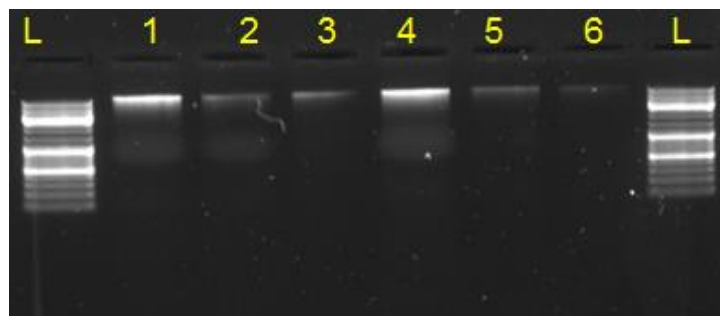


Figure 12 - Genomic DNA extraction. Lanes 1 – 6 represent genomic DNA of transformed colonies.

To determine if the *HST6* gene was deleted, a PCR was performed using three different polymerases. A band of approximately 3800 bp were observed for the six selected colonies as well as for the WT. KAPA Taq PCR Kit v5.13 (KAPABIOSYSTEMS) worked the best where KOD Hot Start DNA Polymerase (Novagen®) showed non-specific amplification. Expand Long Template PCR System

showed poor amplification except for the WT. In lane 12, two bands were observed with a smaller band present at approximately 340 bp which possibly indicates that one of the *HST6* copies were deleted.

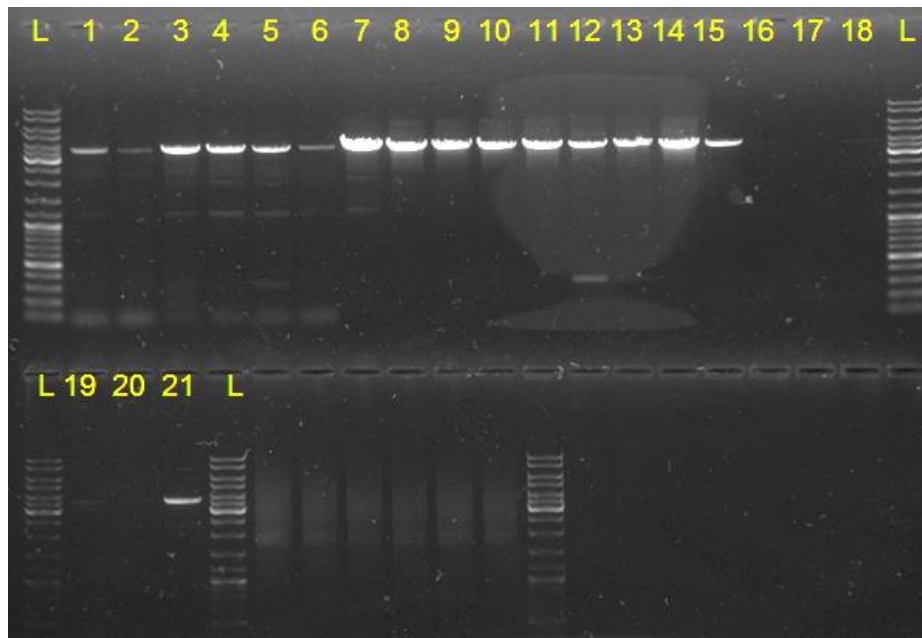


Figure 13 - PCR results. Lanes 1 - 21 represent amplified DNA using primers HST6_Ca-qF1 & Ca_HST6-6R. Lanes 1 – 6 represent a PCR using genomic DNA of transformed colonies with KOD Hot Start DNA Polymerase (Novagen®) and lane 7 represent a PCR using genomic DNA of *C. albicans* (WT). Lanes 8 – 13 represent a PCR using genomic DNA of transformed colonies with KAPA Taq PCR Kit v5.13 (KAPABIOSYSTEMS) and lane 14 represent a PCR using genomic DNA of *C. albicans* (WT). Lanes 15 – 20 represent a PCR using genomic DNA of transformed colonies with Expand Long Template PCR System and lane 21 represent a PCR using genomic DNA of *C. albicans* (WT).

The single colony showing possible deletion of one of the *HST6* copies when grown in YPM media overnight, followed by streaking on YPD and YPD plus 200 $\mu\text{g}.\text{ml}^{-1}$ NTC plates. Nine out of 69 colonies showed absence of growth on the antibiotic plates and indicated that the NTC marker was removed. A second round of transformation was performed on five of these colonies. Resultant colonies (approximately 40 colonies per plate) were picked up and streaked on YPD plates containing 300 $\mu\text{g}.\text{ml}^{-1}$ NTC and

incubated at 37°C overnight. All of the colonies showed growth on the higher concentration of NTC and for confirmation, a colony PCR was performed to confirm *HST6* deletion and Cas9 integration.

Two different reactions were performed for *HST6* deletion confirmation. The first reaction was performed using KAPA HiFi HotStart ReadyMix PCR Kit and the second reaction was performed using Expand Long Template PCR System Kit.

As can be seen from both figures 15 and 16, one band for the transformed colonies was observed of approximately 340 bp (Figure 15 & 16; lane 1-5), which indicated that the transformation was successful as both copies of the *HST6* gene were deleted and the donor DNA was in the place of the ORF. If only one copy was deleted, two bands would have been observed (one of approximately 3 800 bp and one of approximately 340 bp). The WT contained the original *HST6* gene of approximately 3800 bp (Figure 15 & 16, lane 6). It was also confirmed that the Cas9 gene was integrated as a (light) band of approximately 694 bp was obtained (Figure 15 lane 7-11).

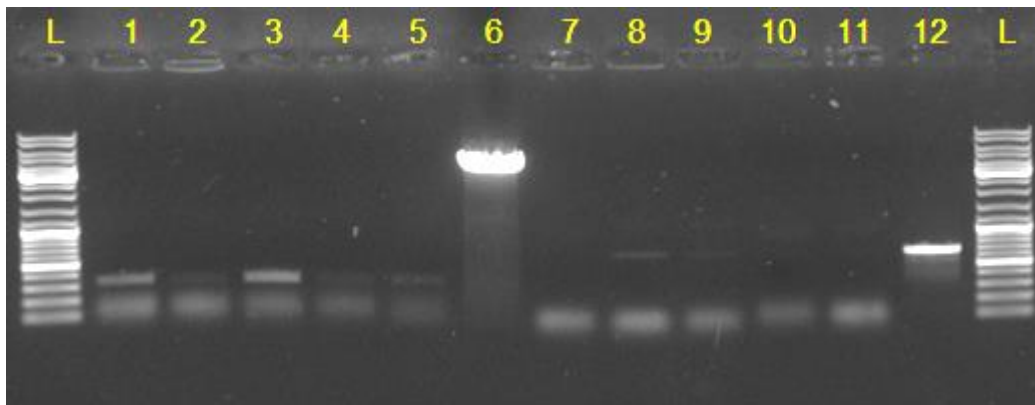


Figure 15 - PCR results. Lanes 1 - 6 represent amplified DNA using primers HST6_Ca-qF1 & Ca_HST6-6R. Lanes 1 - 5 represent amplified DNA using transformed colonies with KAPA HiFi HotStart ReadyMix PCR Kit and lane 6 represent amplified DNA using genomic DNA of *C. albicans* (WT). Lanes 7 – 12 represent amplified DNA using primers CaCas9-1F & CaCas9-1R and lanes 7 – 11 represent amplified DNA from transformed colonies with Expand Long Template PCR System kit. Lane 12 represent amplified DNA using pADH99 plasmid DNA.

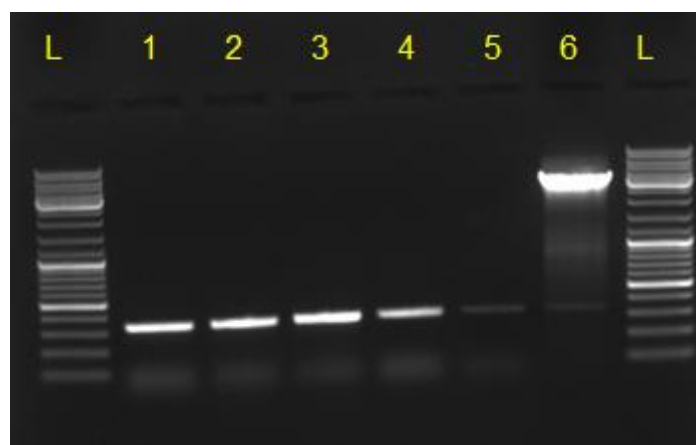


Figure 16 - PCR results. Lanes 1 - 6 represent amplified DNA using primers HST6_Ca-qF1 & Ca_HST6-6R. Lanes 1 - 5 represent amplified DNA using transformed colonies with Expand Long Template PCR System kit and lane 6 represent amplified DNA using genomic DNA of *C. albicans* (WT).

For further confirmation, genomic DNA was extracted using the *Quick-DNA™* Fungal/Bacterial Miniprep Kit (50 preps) (Zymo Research), according to manufacturer's recommendation and bands were observed for transformed colonies (Figure 17).

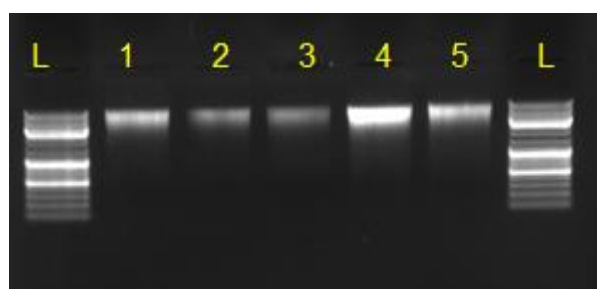


Figure 17 - Genomic DNA extraction. Lanes 1 – 6 represent genomic DNA of transformed colonies.

A PCR was performed using genomic DNA and primers HST6_Ca-qF1 & Ca_HST6-6R. This reaction was performed using KAPA HiFi HotStart ReadyMix PCR Kit.

PCR results obtained using extracted genomic DNA as template (Figure 18) however did not show the 340 bp band but only a 3800 bp band. The latter indicates that the *HST6* gene was still intact. It is uncertain why the colony PCR yielded bands the size of the donor DNA and no bands of the WT gene where the genomic DNA yielded bands of approximately 3800 bp.

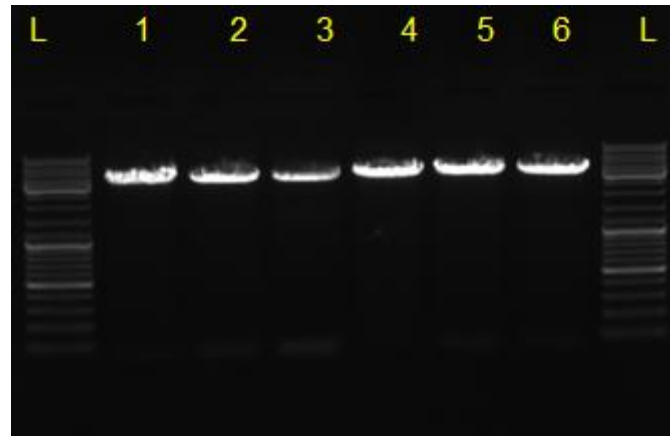


Figure 18 - PCR results. Lanes 1 - 6 represent amplified DNA using primers HST6_Ca-qF1 & Ca_HST6-6R. Lanes 1 - 5 represent amplified DNA genomic DNA of transformed colonies with KAPA HiFi HotStart ReadyMix PCR Kit and lane 6 represents amplified DNA using genomic DNA of *C. albicans* (WT).

To confirm that the 3800 bp band obtained is the *HST6* gene, a restriction enzyme digest was performed using *Bgl*II (Thermo Scientific) according to manufacturer's recommendation. Three bands (approximately 2109 bp, 1265 bp & 434 bp) were observed for all 5 transformed colonies as well as for the WT strain (Figure 19).

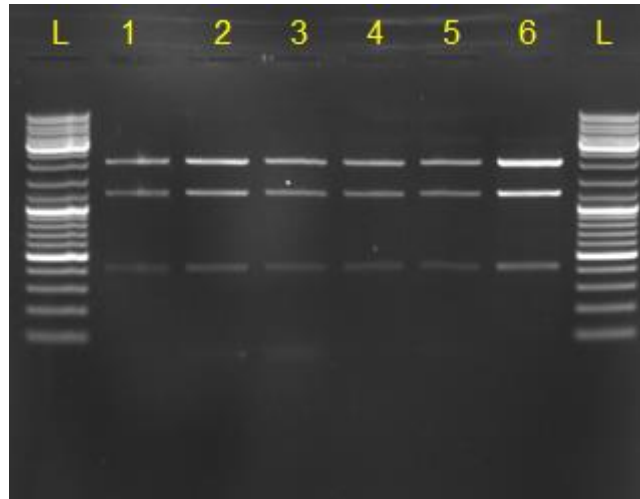


Figure 19 - Restriction enzyme digest results. Lanes 1 - 6 represent digested DNA using *Bgl*I. Lanes 1 - 5 represent digested genomic DNA of transformed colonies and lane 6 represents digested DNA using genomic DNA of *C. albicans* (WT).

4.2 Yeast transformations with the CRISPR-Cas9 construct without the PAM site to remove *HST6*:

When the initial primers for the cloning free stitching was designed (Table 1), the PAM site was included into HST6-CRISPR-1 oligo & HST6-CRISPR-2 oligo primer. New primers were ordered which did not contain the PAM site in the sequence. HST6-CRISPR-3 oligo primer for removal of the *HST6* gene and HST6-CRISPR-4 oligo primer for add-back of the gene was ordered.

The same procedures as previously were followed in creating fragment B (section 4.1), except for the replacement of the HST6-CRISPR-1 oligo primer with HST6-CRISPR-3 oligo primer.

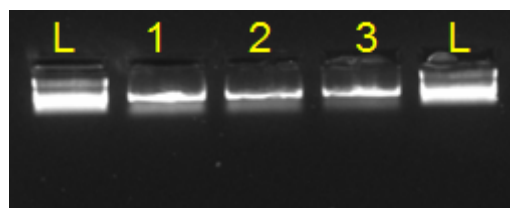


Figure 20 - PCR results. Lanes 1 - 3 represent amplified DNA of Fragment B using primers AHO1097 & HST6-CRISPR-3-oligo.

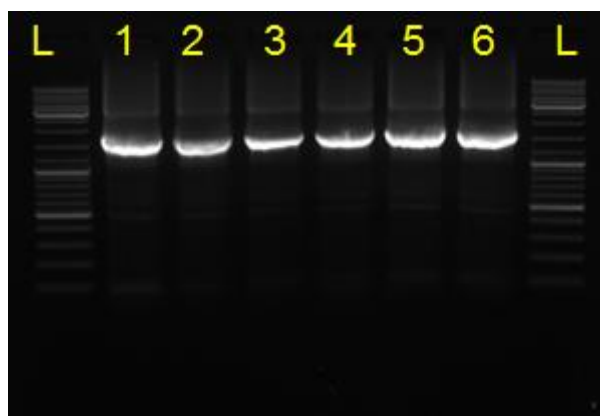


Figure 21- PCR results. Lanes 1 - 6 represent amplified DNA from Fragment A and Fragment B using AHO1237 & AHO1236 primers.

Candida albicans was transformed with *HST6* donor DNA, digested pADH99 and Fragment C (cloning free stitching fragment). Transformed colonies were plated on YPD plates containing 200 $\mu\text{g}\cdot\text{ml}^{-1}$ NTC and incubated at 30°C for 2-3 days. It was observed that the plates with transformed *C. albicans* had growth on all YPD plates containing 200 $\mu\text{g}\cdot\text{ml}^{-1}$ NTC, excluding the negative control. Colonies (98) were streaked out on YPD plates containing 300 $\mu\text{g}\cdot\text{ml}^{-1}$ NTC and 98 colonies were observed after overnight incubation at 37°C.

For confirmation, a colony PCR was performed on six randomly selected colonies. Primers HST6_Ca-qF1 & Ca_HST6-6R with KAPA Taq PCR Kit v5.13 (KAPABIOSYSTEMS) was used to amplify the DNA sequences.

PCR results showed that all six colonies obtained the band of approximately 337 bp, which, indicated that both copies of the gene were deleted (Figure 23). It also showed that the Cas9 gene was transformed successfully for all colonies.

A genomic DNA extraction was performed on the six colonies using the ZR Fungal/Bacterial DNA MiniPrep kit according to manufacturer's instructions (Zymo Research) (Figure 22). A second PCR was performed to confirm *HST6* deletion using KOD Hot Start DNA Polymerase (Novagen®). This PCR reaction indicated that five of the six colonies contains the WT gene, and only one colony had both copies of the gene removed (Figure 23, Lane 5).

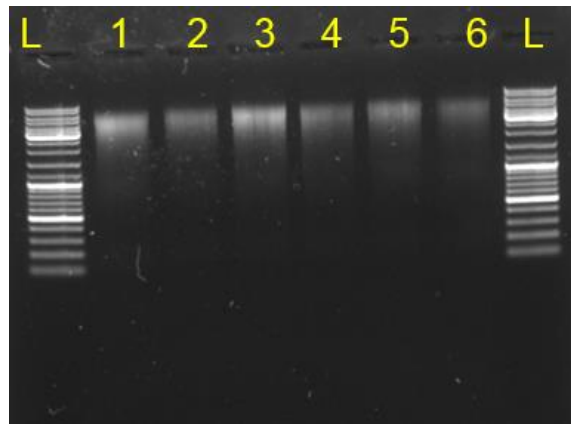


Figure 22 - Genomic DNA extraction. Lanes 1 – 6 represent genomic DNA of transformed colonies.

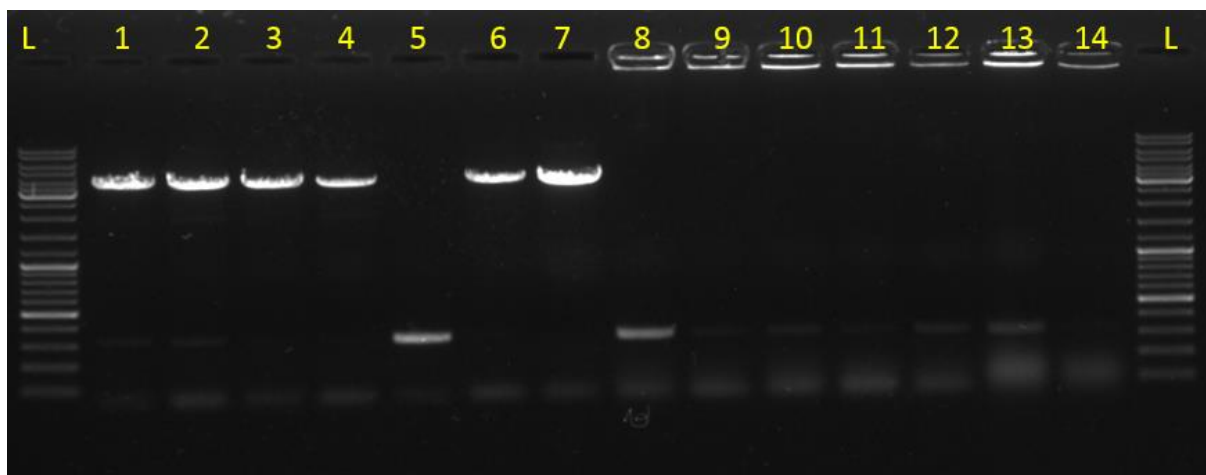


Figure 23 - PCR results. Lanes 1 - 6 represent amplified DNA from genomic DNA and lanes 7 – 14 represent amplified DNA from whole cells. Lanes 1 and 7 represent the WT. Lane 5 indicates that both copies of the *HST6* gene are removed as only one band is observed.

Following confirmation that both copies of the *HST6* gene was deleted, the gene had to be added back into the genome of *C. albicans*. Donor DNA was constructed by amplifying the *HST6* ORF including approximately 500 bp up-and downstream regions, (Figure 24).

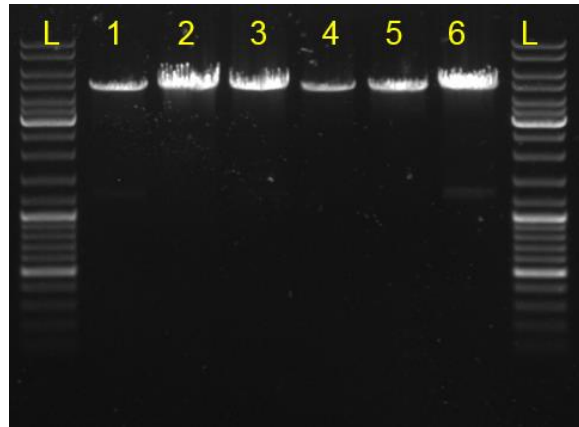


Figure 24 - PCR results. Lanes 1 - 6 represent amplified DNA (*HST6* ORF) from genomic DNA of *C. albicans* SC 5314.

In order to add the gene back, the NTC marker had to be removed from the *hst6* deletion strain. Following growth in YPM media and streaking on YPD plates with or without NTC, colonies that showed growth on YPD plates and not on NTC plates were picked and inoculated for transformation.

Cells were transformed with *HST6* donor DNA (*HST6* ORF) (Figure 23), digested pADH99 and Fragment C (cloning free stitching fragment) obtained after linking Fragment A and B (Figure 24) through a cloning free stitching PCR reaction. This was plated out on YPD plates containing 200 $\mu\text{g}\cdot\text{ml}^{-1}$ NTC and incubated at 30°C for 2-3 days. Resultant colonies (23) were picked up and streaked out on YPD plates containing 300 $\mu\text{g}\cdot\text{ml}^{-1}$ NTC and incubated at 37°C overnight. All 23 colonies grew on the higher concentration of this antibiotic.

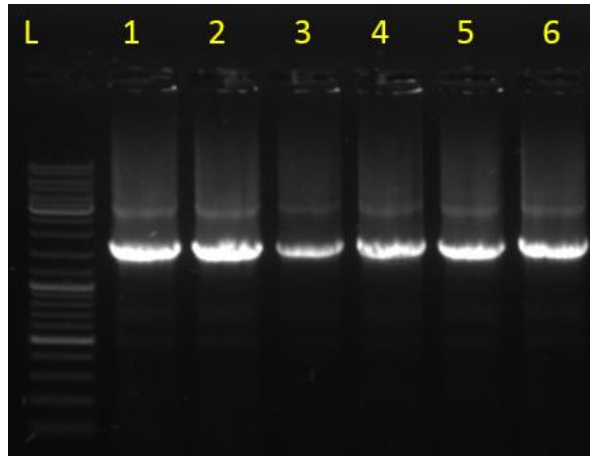


Figure 25 - PCR results. Lanes 1 - 6 represent amplified DNA from Fragment A and Fragment B using AHO1237 & AHO1236 primers.

A genomic DNA extraction was performed on colonies using the ZR Fungal/Bacterial DNA MiniPrep kit according to manufacturer's instructions (Zymo Research). A PCR was performed to confirm *HST6* deletion using KAPA HiFi HotStart PCR Kit (KAPABIOSYSTEMS). Primer sets HST6_Ca-qF1 & Ca_HST6-6R and Ca_4F + NcoI and Ca_HST6-2R was used. It was observed that 4 out of 6 colonies still had both copies of the gene removed (Figure 26) (lanes 1 – 8). However, lane 10 and 11 indicated band size corresponding to size expected if the *HST6* gene was re-introduced to the genome.

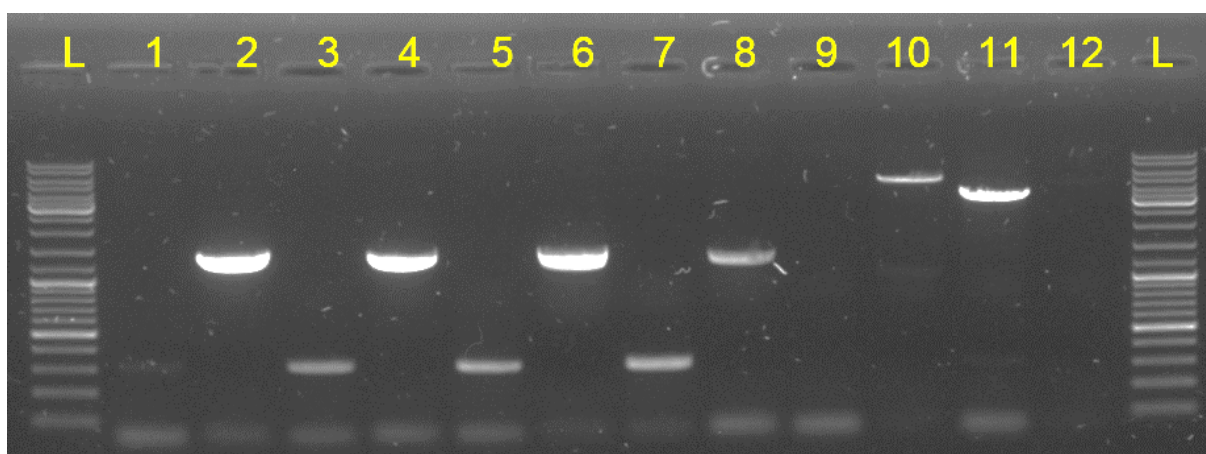


Figure 26 - PCR results. Lanes 1, 3, 5, 7, 9 & 11 represent amplified DNA using primers HST6_Ca-qF1 & Ca_HST6-6R. Lanes 2, 4, 6, 8, 10 & 12 represent amplified DNA using primers Ca_4F + NcoI and Ca_HST6-2R.

For further confirmation these PCR products (Figure 26, lane 10 and 11) were digested using *Bgl*II, *Hind*III and *Xba*I (Figure 27). Restriction profile obtained confirmed that the *HST6* gene was reintroduced into the genome of the *hst6* double deletion mutant.

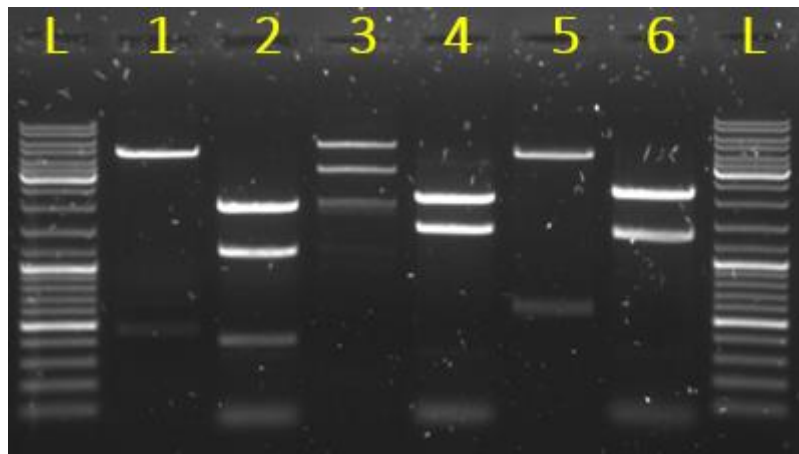


Figure 27 - Restriction enzyme digest results. Lanes 1, 3 & 5 represent digested DNA of the PCR product obtained with primers HST6_Ca-qF1 & Ca_HST6-6R (Figure 26, lane 10). Lanes 2, 4 & 6 represent digested DNA of the PCR product obtained with primers Ca_4F + *Nco*I and Ca_HST6-2R (Figure 26, lane 11). Lanes 1 and 2, restriction analysis using *Bgl*II. Lanes 3 and 4 using *Hind*III and lanes 5 and 6 using *Xba*I.

4.3 Phenotypic changes

The wild type, double deletion and complemented strains were grown in YPD broth. A dilution series was made and 10 μ l of the diluted cells pipetted onto YPD plates. As can be observed from Figure 28, a phenotypic change occurred between the WT and double deletion mutant after incubation at 37°C for 2 days. Colonies representing the WT were smooth in comparison to the double deletion mutant that were wrinkled. The strain with the added back gene was smooth and looked like that of the WT. It was also observed that, when harvesting cells of the double deletion mutant using centrifugation, the pellet was less compact than that of the WT and the complemented strain. It was also observed that the colonies of the double deletion mutant became

more wrinkled at higher dilutions, this might be due to the effect of quorum sensing, but further investigation needs to be performed.



Figure 28 - Dilution series results. The first column represents cells from the WT. The second column represents cells from the double deletion mutant and the third column represents cells from the mutant with the added back gene. From top to bottom, the dilution was as follows: undiluted, 10^{-1} , 10^{-2} , 10^{-3} & 10^{-4} .

Microscopic analyses of these cells indicated that both the WT (Figure 29, A) and the complemented strain (Figure 29, C) are in the yeast form, whereas the cells of the double deletion mutant (Figure 29, B) are mostly pseudohyphae and true hyphae.

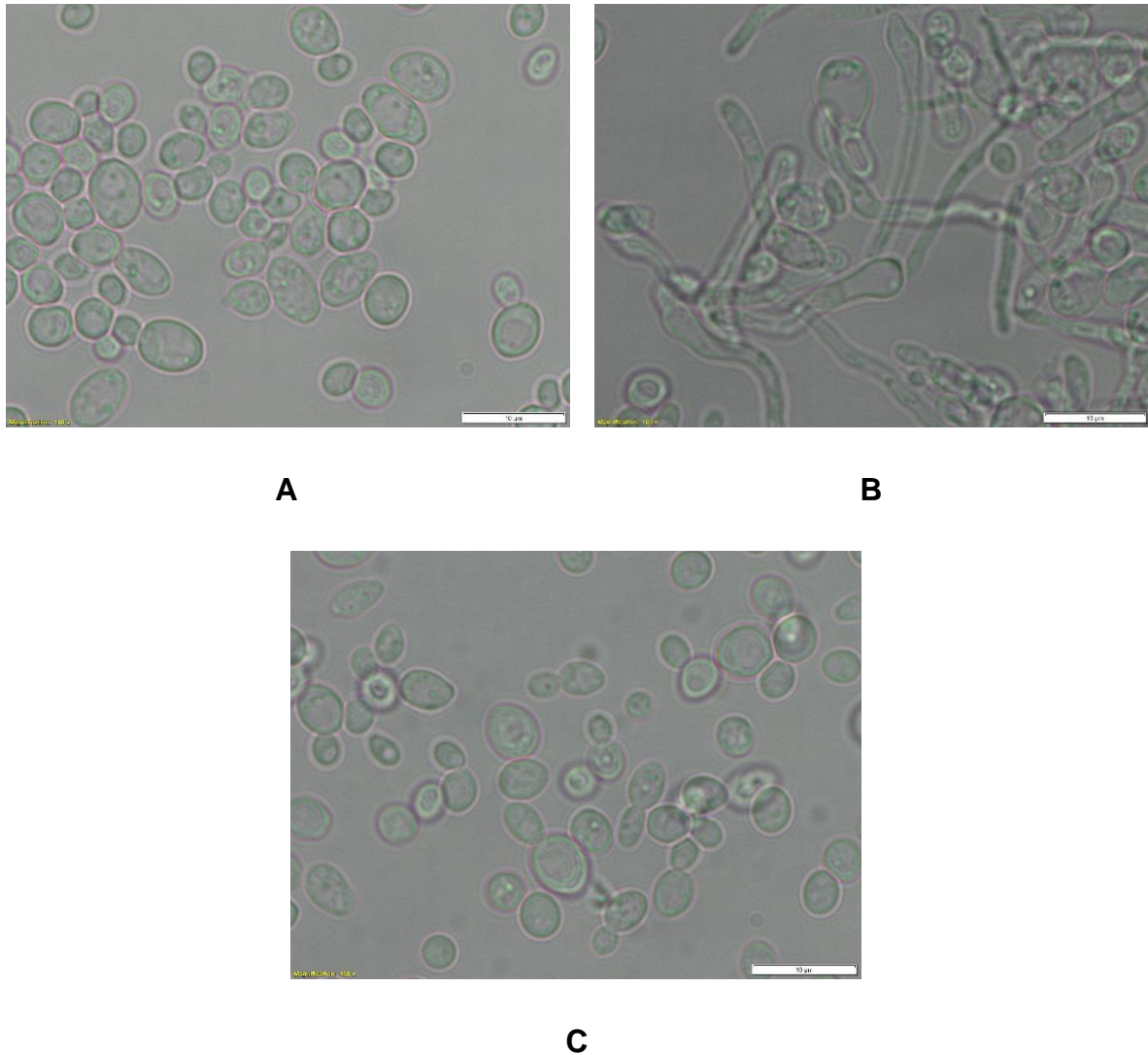


Figure 29 – Microscopic Results. A - Microscopic image of the WT cells at a 400x magnification. B - Microscopic image of the double deletion cells at a 400x magnification. C - Microscopic image of the complemented strain at a 400x magnification.

4.4 Fluconazole sensitivity of biofilms

Results from the XTT assay revealed that WT biofilm metabolic activity was reduced by 56% in the presence of 1 mg.l⁻¹ fluconazole (Figure 30). The double deletion mutant showed a significant ($P = 0.0009$) increase in reduction to 75%. These results suggest that *HST6* may play a role in fluconazole susceptibility. However, this reduction was reduced to 70% in the complemented strain, but was still significantly ($P = 0.0066$) higher than that of the WT.

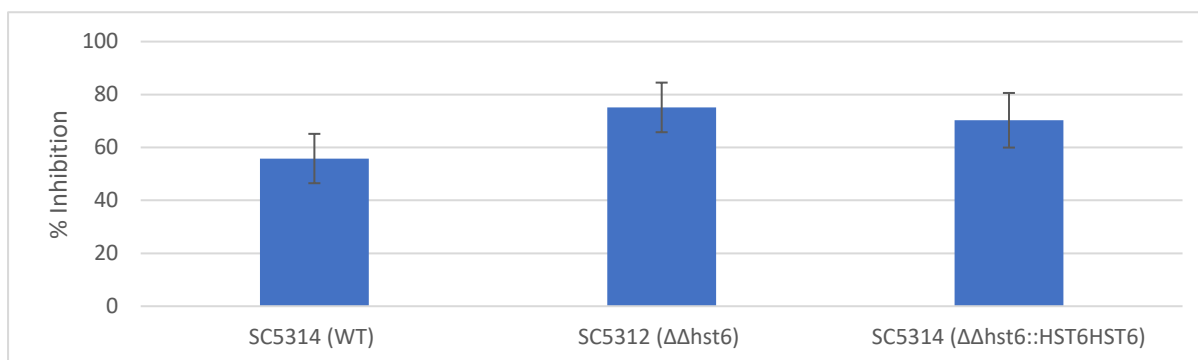


Figure 30 - Effect of deletion of *HST6* on fluconazole susceptibility

This failure to reinstate the WT phenotype in the complemented strain may be due to unwanted off-target mutations that can be induced when using the CRISPR-Cas9 gene editing system (Kleinstiver *et al.*, 2016) or that $\Delta\Delta hst6$ cells were lost during the washing step as this mutant does not adhere well to the plastic. The former is however unlikely seeing that *C. albicans* can repair breaks in the DNA strand very efficiently. For future work, this experiment can be repeated more than three times to eliminate technical errors.

5. Conclusion

After various attempts to create gene deletions in *Candida albicans*, it was confirmed that a double deletion mutant of *HST6* was constructed in addition to a complemented strain was also constructed. This was all confirmed via the polymerase chain reaction (PCR) as well as through observation of phenotypic changes.

A limiting factor experienced, includes the addition of a PAM site in the overlap primer with the CRISPR insertion site. This led to colonies being obtained after transformation, which indicated successful integration, but allowed for continuous cleaving and repair of the donor DNA, which led to false positives. New primers were designed without included PAM sites, new fragments were amplified, and

transformation was performed again. Colonies were observed, PCR was performed, and successful gene deletion was obtained.

For the complementation of the deleted gene, the Nourseothricin resistant marker had to be removed. Some struggles were experienced as a lawn of cells were obtained after transformation and not single colonies as expected. This was corrected by growing cells for about 48 hours in maltose and not overnight as before. Transformation was performed again, and single colonies were obtained. PCR confirmation confirmed the removal of the deletion construct including the Nourseothricin resistant marker. Following a second round of transformation both PCR and restriction analysis using three different restriction enzymes confirmed the construction of a complemented strain.

Morphological differences were observed between the different strains. The wild type is more yeast like, whereas the double deletion mutant produced mainly pseudohyphae and true hyphae, however the complemented strain was also in the yeast form. These morphological differences were also evident in the colony appearance, with the wild type and complemented strain growing as smooth colonies and the colonies of the double deletion mutant exhibiting a wrinkled appearance. It was also observed that in a cell suspension, the double deletion mutant formed clumps/ flocs, not seen with the wild type and complemented strain. When cells are harvested via centrifugation, the cell pellet of the wild type and the complemented strain is more stable and did not come resuspended when the supernatant is discarded. In the case of the double deletion mutant, the pellet is unstable and the cells resuspended when the supernatant was discarded. In addition, results obtained from exposure of the different strains to fluconazole suggests that *HST6* may contribute to fluconazole resistance in *C. albicans*, although the presence of off target effects and technical issues may have hampered confirmation of the phenotype in the complemented strain. These results strongly suggest that *HST6* is involved in the yeast to hyphae switch (an important virulence factor) and possibly resistance towards fluconazole. Both these phenomena warrant further investigation, since they play important roles in clinical manifestations and treatment of infection.

To conclude, we have implemented an optimised CRISPR-Cas9 gene editing system in *Candida albicans* and managed to create a double deletion mutant of *HST6*. We have also constructed a complemented strain where *HST6* was re-introduced into the genome of the organism. This will allow for various genes to be deleted in *C. albicans* and will lead to the determination of the function of several genes whose function is still unknown. More information will become available on drug resistance of *C. albicans* which might help to solve this problem in the future.

6. References

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Chapter 5

Summary

Summary

The aim of this project was to construct a *HST6* double deletion mutant of *Candida albicans* to elucidate the role and function of this gene. *HST6* is an ABC transporter and homologue of *Ste6* (*Saccharomyces cerevisiae*) which plays a role in mating in this organism. The first method used was the *SAT1* flipper system, which alters genes using an antibiotic resistant marker (Nourseothricin) to select for integration. Homozygous mutants are created by transforming the organism with DNA containing the FLP gene which are flanked by target sequences found in the genome of *C. albicans*. Two rounds of integration are required as this organism has a duplicate set of genes and both copies need to be removed sequentially. For the second round of integration, the mutant is grown on Maltose to flip out and regenerate the nourseothricin resistant marker. In this study a previously constructed plasmid was used to delete the *HST6* gene, but after various attempts, no success was achieved. Transformations were unsuccessful and after molecular confirmation protocols such as PCR was performed, the gene remained intact. While using the *SAT1* flipper system, the article by (Vyas *et al.*, 2015) was published and the CRISPR-Cas 9 method was used for gene deletion in *C. albicans*. This system relies upon the use of the endonuclease (Cas9) and guide RNA to create double stranded cleaving. This system wasn't available to order, and so a similar system was constructed. While in the process to develop this system, another article was published, describing a CRISPR-Cas gene editing system. This system was however made available to the wider scientific community through depositing of all plasmids at Addgene (www.addgene.org). This system was subsequently obtained via Addgene and a proof of concept, the *ADE2* gene was targeted as the double deletion mutant showed an easily observed phenotype where if both alleles are deleted, colonies turn from white

to red. After several attempts, a double deletion mutant of *ADE2* was constructed in *C. albicans* using the CRISPR-Cas9 system and red colonies were observed. This allowed for optimisation of the system which led to the *HST6* gene to be targeted. After confirmation via PCR, the *HST6* gene was removed and it was managed to create a complemented strain where the *HST6* gene was added back into the genome of the organism. The wild-type strain, double deletion mutant and complemented strain was used to determine the influence of fluconazole on the different variants of the organism. The XTT assay revealed that the biofilm metabolic activity of the wild type strain was less influenced by fluconazole (55.8% reduction) as the biofilm metabolic activity of the mutants (75.1 % and 70.3 % respectively). It can be assumed that *HST6* plays a role in fluconazole resistance, but further investigation is required as no significant difference was obtained between reduction in biofilm metabolic activity because of fluconazole addition, between the double deletion mutant and the complemented strain. Nonetheless, a CRISPR-Cas9 gene editing system was used effectively to construct both a double deletion mutant of *HST6* as well as a complemented strain in the diploid *C. albicans*. This system will allow for more genes to be targeted in the future and might lead to a solution to drug resistance in *C. albicans*.