Phenotypic Characterisation of *Candida albicans* Mutants with Deletions of Arachidonic Acid Responsive Genes

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Dedicated with love, humbleness and supreme respect, to Mbuti, Nnana, Tshidi (and precious daughter - Tshwarelo), Ratu, and late grandparents, uncle and friends.

To Ghost!

“"It is important to realize that our inability to answer a question says nothing about whether the question itself has an answer. Exactly how many people were bitten by mosquitoes in the last sixty seconds? How many of these people will contract malaria? How many will die as a result? Given the technical challenges involved, no team of scientists could possibly respond to such questions. And yet we know that they admit of simple numerical answers. Does our inability to gather the relevant data oblige us to respect all opinions equally? Of course not. In the same way, the fact that we may not be able to resolve specific moral dilemmas does not suggest that all competing responses to them are equally valid" _Sam Harris._"
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Chapter I – *Candida albicans* Mutant Construction and Characterization of Selected Virulence Determinants

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1. **Motivation**

The human fungal pathogen, *Candida albicans*, is diploid and grows as budding cells which can extend to the mycelial state under favorable conditions (Kurtzman, 2011). In addition, *Candida albicans* is an obligate diploid organism, requiring disruption of two alleles to generate a null mutant used to analyze phenotypes contributing to virulence. It is also readily isolated from clinical samples due to its opportunistic lifestyle (Cooper Jr, 2011), and, unlike non-pathogenic yeasts, is less frequently isolated from the environment outside its host i.e. leaves, fruit surfaces, exudates, sugar-rich source dumps, sewage sludges, and insects. As a pathogen, it accounts for a large number of mucoses (>60%) occurring in the digestive tract, mucocutaneous tissues and skin as well as in the bloodstream. Individuals infected are usually immune deficient and/or immune suppressed due to medical interventions or diseases such as AIDS or other critical illnesses. The success of *C. albicans* to cause infections is largely attributed to a number of virulence factors including acquired resistance towards antimicrobial drugs and, to a broad extent, its polyphenic nature which allows it to navigate through the dynamic host environmental conditions such as varying temperatures, pH, stress (e.g. oxidative, nitrosative, osmotic, heavy metal and cell wall stress), carbon dioxide levels and available nutrient sources (e.g. carbon, nitrogen, phosphorus or sulphur sources). In addition, specific virulence factors such as attachment to the human mucosa and secretion of lipolytic or proteolytic enzymes further contribute to the nature of *C. albicans* to invade host tissues and cause devastating infections (Franz et al., 1998; Calderone & Fonzi, 2001; Martinez et al., 2002; Khan et al., 2010; Sullivan & Moran, 2011; Huang, 2012; Nobile et al., 2012).

Research conducted on fungal oxygenated lipids, also known as oxylipins, has revealed that cultures of *C. albicans* can utilize exogenously supplemented arachidonic acid (AA), a polyunsaturated omega-6 fatty acid, in a mammalian culture medium such as RPMI-1640, and produce various eicosanoids such as prostaglandin E$_2$ (PGE$_2$) (Deva et al., 2000). Although AA does not form part of the lipid component of yeasts, in a mammalian host, *C. albicans* can evoke mechanisms needed for the liberation of AA from nearby affected tissues and produce the immunomodulatory molecule, PGE$_2$ (Suram et al., 2010). Production of this metabolite regulates numerous aspects of inflammation and operations carried out by the immune system (Kalinski, 2012). In fungal pathobiology, PE$_2$ is also known to regulate morphogenesis by stimulating germ tube production in *C. albicans* and the closely related species, *C. dubliniensis* (Kalo-Kelin & Witkin, 1990; Ells et al., 2011), suggesting that this eicosanoid is also an important virulence factor. However, research on fungal AA metabolism is still emerging and PGE$_2$ biosynthetic pathways in pathogenic yeast still remain to be understood. So far, several lines of evidence indicate that PGE$_2$ can trigger a unique gene expression pattern in the genome of *C. albicans* (Levitin & Whiteway, 2007). The effect of this transcriptional activation also suggests that *C. albicans* not only triggers production of, but also senses, PGE$_2$ in its surrounding environment (Levitin & Whiteway, 2007). An independent study carried out in our research group indicates that AA, the precursor for PGE$_2$, also activates a
unique transcriptional programme (Ells, 2011). This transcriptional response was induced in a \textit{C. albicans} clinical strain (NRRL Y27077) isolated from a patient with skin infection. In this response, significant up-regulation of several important genes including those encoding membrane-bound proteins, cell wall maintenance and pH associated proteins was observed. Intriguingly, this transcriptional profile did not reveal up-regulation of genes currently known to be directly involved in PGE$_2$ production. Therefore, the current work aims to understand the role of some of the genes activated during conversion of AA into PGE$_2$ and to decipher whether they are directly or indirectly involved in this process, and ultimately how they contribute to virulence of \textit{C. albicans}. Because this microarray data was generated in the background of \textit{C. albicans} NRRL Y-27077, work in this study was also continued using the same strain.

2. **Introduction**

\textit{Candida} species reproduce asexually by budding cells which can switch to mycelia. Mycelial state comprises of pseudo- (chain of yeast cells that is intermediate between connected budding cells; no discernible septa and the terminal cell is shorter than or long as the preceding immediate cells) and/or true hyphae (refractive, straight septa and terminal cell is longer than preceding immediate cells) (Sudbery et al., 2004; Bennett, 2010; Cooper Jr, 2011; Kurtzman, 2011). \textit{Candida} species belong to a class of ascomycotan fungi (Saccharomycetes) which include the model yeast, \textit{Saccharomyces cerevisiae}. There has been an interest in studying \textit{Candida} species, especially those classified as human fungal pathogens due to clinical or medical concerns (e.g. \textit{Candida albicans}) and those recognized as emerging pathogens (e.g. \textit{C. dubliniensis}, \textit{C. glabrata}, \textit{C. krusei}, \textit{C. metapsilosis}, \textit{C. orthopsilosis} and \textit{C. parapsilosis}). Although these pathogenic yeasts commensally colonize the digestive tract, mucocutaneous tissues and skin of humans as well as organs of other warm blooded animals, they can cause diseases through infection in these sites (collectively called candidiasis) or spread via the bloodstream (candidemia) to other internal organs (invasive candidiasis) (Rueping et al., 2009; Alby & Bennett, 2010; Bennett, 2010; Cooper Jr, 2011). Therefore, most recorded infections result from the same commensal strains that colonize the host but have a triggered pathogenic state due to the impaired or damaged immune response (Pérez et al., 2013; Kavanaugh et al., 2014; Zaborin et al., 2014).

Common risk factors which amount to \textit{Candida} infections include frequent use of broad-spectrum antimicrobial therapy, subjection to radio-therapy, abdominal surgery, endocrine disorders, haematological malignant diseases and transplantation as well as an immunodeficient host or critical illness (Alby & Bennett, 2010; Mikulska et al., 2011). Predisposing conditions leading to infections by specific \textit{Candida} species include extensive flucanazole use, presence of cancers, old age, application of vascular catheters and aggressive surgery (\textit{C. glabrata}), neutropenia and bone marrow transplantation (\textit{C. krusei} and \textit{C. tropicalis}), previous use of polyenes (\textit{C. guilliermondii} and \textit{C. lusitaniae}) as well as intravenous nutrition or artificial supply of nutrients (hyperalimentation) (\textit{C. parapsilosis}) (Mikulska et al., 2011). As a result, \textit{Candida} species are the fourth most common cause of hospital-acquired (nosocomial) blood stream infections in North America, accounting for 8-10% of such infections and resulting in the annual cost of treatment of up to $1 billion and mortality rate of 50% yearly (Pfaller & Diekema, 2007; Rueping et al., 2009).
Furthermore, *C. albicans* is responsible for 40% of device-associated infections in the United States of America due to biofilms forming on medical devices and their inherent resistance to antimicrobial therapy (Wenzel, 1995; Douglas, 2002, 2003; Kojic & Darouiche, 2004; Pfaller & Diekema, 2007; Rueping et al., 2009). In the European Union (EU) alone, the occurrence of *Candida* infections is as low as 5-7% while in Brazil, about 3.7% cases of candidemia have been reported in healthcare facilities (Hajjeh et al., 2004; Colombo et al., 2006).

Research regarding opportunistic lifestyles of pathogenic yeasts such as *C. albicans* is directed towards understanding phenotypic traits and underlying genetic mechanisms required to thrive and cause infections in the host. The contribution of these pathogenic aspects to the overall virulence of *C. albicans* has received comprehensive research attention and undergone scrutiny (Calderone & Fonzi, 2001; Schaller et al., 2005; Karkowska-Kuleta et al., 2009). Phenotypic switching and underlying switch programmes, adhesion to mucosal membrane and secretion of hydrolytic enzymes, are some of the well studied virulence determinants in *C. albicans* infections. Furthermore, extracellular polymeric matter (matrix) that develops to embed *C. albicans* cells (collectively known as biofilms) is the main cause of device-associated (iatrogenic) infections in the clinical settings (Wenzel, 1995; Douglas, 2002, 2003; Kojic & Darouiche, 2004).

Independent research groups, including our own, have closely analyzed other virulence factors such as the ability of *C. albicans* to utilize distinct carbon sources such as arachidonic acid (AA), a 20-carbon polyunsaturated omega-6 (ω-6) fatty acid. When supplemented from the external source or released from damaged or infected host cells such as macrophages (Castro et al., 1994; Suram et al., 2010), AA serves as a carbon source for cellular growth of *C. albicans* as well as a precursor for the production of eicosanoids [e.g. prostaglandin $E_2$ (PGE$_2$)] (Deva et al., 2000; Noverr et al., 2001, 2003; Ciccoli et al., 2005; Ells et al., 2009, 2011; Shareck & Belhumeur, 2011). In mammals, PGE$_2$ is synthesized via the cyclooxygenase (COX) and prostaglandin synthase pathways. In addition, PGE$_2$ plays many biochemical roles in pain, fever (Ootsuka et al., 2008), inflammatory disorders (Funk, 2001; McCoy et al., 2002; Chizzolini & Brembilla, 2009), homeostasis (Lundequist et al., 2010), thrombosis (Gross et al., 2007) as well as allergic and immune responses (Nagamachi et al., 2007). As a metabolite in pathogenic yeasts, PGE$_2$ assumes a pivotal role in virulence because it drives germ tube and biofilm development in *C. albicans* (Kalo-Klein & Witkin, 1990; Noverr & Huffnagle, 2004; Ells et al., 2011). This is despite the fact that AA, the precursor for PGE$_2$, does not form part of the lipid component in yeasts. How PGE$_2$ elicits the transition from budded to hyphal cells is still an open question, although there are suggestions that this may occur via cAMP-dependent signalling (Kalo-Klein & Witkin, 1990).

Gene knock-out studies contribute largely to constructed mutant libraries utilized by researchers to study the genetic components underlying virulence of *C. albicans* (Homann et al., 2009; Fox & Nobile, 2012; Lohse et al., 2013; Nobile et al., 2012; Guan et al., 2013; Pérez et al., 2013). Since construction of these libraries, it is now possible to conduct large-scale screening of genetic determinants and their effect on the biology of *C. albicans* and the interplay with the mammalian host. Mutant libraries and their underpinning techniques should also be considered for employment in PGE$_2$ research since they can offer a reasonable genetic explanation regarding
important regulatory circuits modulating PGE\textsubscript{2} production or those modulated by the metabolite itself. So far, very few studies have analyzed strains defective in morphogenetic pathways in the presence of AA or PGE\textsubscript{2} (e.g. Alem & Douglas, 2005; Erb-Downward & Noverr, 2007; Erb-Downward et al., 2008). One such study was by Levitin and Whiteway (2007) where they uncovered regulatory pathways that rely on PGE\textsubscript{2} for signaling. An exciting possibility is the screening of a kinase library (Blankenship et al., 2010) and transcription regulator library (Homann et al., 2009) using AA to determine which regulatory pathways are involved in PGE\textsubscript{2} production. In this literature review, some of the strategies used to compile mutant libraries used to study \textit{C. albicans} virulence traits and what has been achieved so far by studying these libraries is discussed.

3. \textit{Candida albicans} as a human fungal pathogen

\textit{Candida albicans} is a major contributor to local infections such as cutaneous, genital, oesophageal and oral thrush as well as systemic infections in immunocompromised and immunosuppressed individuals. This yeast is not readily isolated from the environment but frequently isolated from clinical samples due its opportunistic lifestyle (Cooper Jr, 2011). One possible explanation why \textit{C. albicans} is not so common in land habitats (e.g. aquatic environments contaminated with sewage effluents, human or animal excrement) might be that it has lost the ability to compete with land microbes such as \textit{Bacillus} species and predators such as amoebas and molds (Cook & Schlitzer, 1981).

\textit{Candida albicans} accounts for circa 62\% of candidiasis cases (Miller & Mejicano, 2001) because of several key factors. These include acquired resistance to antimicrobial drugs observed in isolates obtained from HIV patients (Franz et al., 1998; Martinez et al., 2002; Pfaller et al., 2008). This virulence factor can be attributed to mutations in the drug targets which usually causes a defect in binding of antimicrobial drugs. More often, overexpression of the multidrug transporters from the ATP-binding cassette (ABC) family of proteins, Cdr1p and Cdr2p, has largely been implicated in antifungal drug resistance such as azoles. This overexpression has been typified by the rate of mutations in the transcription factor, Tac1p (Transcriptional Activator of CDR genes) (Siikala et al., 2010).

Another important virulence factor which facilitates colonization of \textit{C. albicans} into the host important tissues is phenotypic switching, also known as morphological transition, indicated in Fig. 1. This can be a transition from the default white to the mating competent opaque phase (white-opaque switching) or a transition from the yeast (typical white state) to the hyphal phase (yeast-hypha switching) under favorable conditions. White-opaque switching occurs in strains homozygous at the mating type locus (\textit{MTL}) i.e. \textit{MTLa} or \textit{MTLa} strains (Hull & Johnson, 1999; Miller & Johnson, 2002), and is regulated by the White-Opaque Regulator 1 protein (Wor1p) (Huang et al., 2006; Zordan et al., 2006, 2007; Lohse & Johnson, 2009; Morschhäuser, 2010; Soll, 2014; Tao et al., 2014a). Unlike white cells, opaque cells are poor colonizers of the \textit{in vivo} infection model but better colonizers of the \textit{ex vivo} model where they may express opaque-specific proteolytic enzymes and result in skin cavitations (Kvaal et al., 1999; Soll, 2014). Typical white cells are \textit{MTLa/α} in which Wor1p is repressed by the a1-a2 heterodimer (Hull & Johnson, 1999; Miller & Johnson, 2002; Huang et al., 2006; Zordan et al., 2006). During this repression, the
white cell formation transcriptional regulator, Enhanced Filamentous Growth 1 protein (Efg1p), becomes active and stabilizes the white phase. Recently, white cells, particularly those of the “a” mating type, have been shown to facilitate opposite- and same-sex mating in opaque cells (Tao et al., 2014b) (Fig. 1). Yeast to hyphal switching is regulated by many transcription factors including the Cph1p, Efg1p and the inducer of hyphal genes (Tec1p) whose promoter is bound directly by *Candida* Pseudo-Hyphal regulator 2 protein (Cph2p) (Biswas et al., 2007; Huang, 2012; Guan et al., 2013; Si et al., 2013). In contrast to white cells, opaque cells require unique filamentation programmes (Si et al., 2013) in which Biofilm-Cell wall Regulator 1 protein (Bcr1p) plays a central role (Guan et al., 2013) (Fig. 1). Both the yeast and hyphal cells have been observed in sites of infections (Felk et al., 2002), suggesting that morphological transition is crucial for colonization and causing infections (Huang, 2012). Under specific host conditions such as stress or starvation, phenotypic switching can contribute to biofilm formation. For instance, biofilm formation on medical devices requires regulation of yeast-hyphal transition (Nobile & Mitchell, 2005; Nobile et al., 2006; Uppuluri et al., 2010) while pheromones secreted by opaque cells (Miller & Johnson, 2002) contribute to biofilm thickness due to expression of pheromone receptors also found on white cells (Daniels et al., 2006). While opaque cells can signal biofilm formation in white cells (Daniels et al., 2006), biofilms promote efficient mating, firstly, by providing protection to the very few present opaque cells to mate and secondly, by stabilizing pheromone gradients (chemotripsin) between mating competent partners (Daniels et al., 2006; Ene & Bennett, 2014). Biofilm formation is regulated by the Bcr1p (Fig. 1), which in turn regulates different adhesins (e.g. Als1p and Als3p) and cell wall proteins (e.g. Ece1p and Hwp1p) (Nobile & Mitchell, 2007; Nobile et al., 2006a, b, 2009, 2012; Guan et al., 2013). On the other hand, biofilm matrix formation is negatively regulated by the Zap1p/Csr1p (*Candida* Suppressor of Rok1p protein) (Nobile et al., 2009). The zap1/zap1 delta strains produce over 3-fold more soluble β-1,3 glucan (a polymer associated with extra cellular matrix in biofilms), in vivo, compared to the wild type strain (Nobile et al., 2009). In contrast to white cells, *C. albicans* opaque cells form unstable biofilms which can easily revert to white cells (Daniels et al., 2006).

The environment plays a crucial role in stimulating signaling pathways in *C. albicans* hence different environmental cues are routinely analyzed in conjunction with these pathways (Fig. 1). For instance, signaling of white-opaque switching is induced by levels of CO₂ (1-5%) and N-acetyl glucosamine (GlcNAc) (1-2%) as well as physiological temperatures (25-37 °C) (Huang et al., 2009, 2010; Huang, 2012). In addition, opaque cells form conjugation tubes and undergo mating in response to pheromone treatment and this may increase genetic fitness and drug resistance in the resulting population of cells while pheromone treatment induces biofilm thickness in white cells (Ene & Bennett, 2014). Signaling of the yeast-hypha transition in white cells is induced in serum, pheromone, GlcNAc or glucose as well as at physiological levels of CO₂ (5%) via the Ras1/cAMP pathway (Huang, 2012; Lin et al., 2013). Efficient filamentation in the opaque phase is induced by unique environmental conditions such as growth on sorbitol and minimal medium with or without amino acids or with a low phosphate source as well as 1.25% GlcNAc at 25 °C (Si et al., 2013) (Fig. 1).
Fig. 1. Regulation of phenotypic switching and virulence in *C. albicans*. Phenotypic switching is triggered and regulated by availability of nutrient signals in the surrounding environment.
morphological defects of these mutant strains is still not determined. Furthermore, exogenously supplemented PGE$_2$ has been shown to up-regulate genes coding for proteins involved in cell cycle and cell growth, protein degradation and sterol metabolism while down-regulating those involved in budding, carbohydrate metabolism and fatty acid biosynthesis (Levitin & Whiteway, 2007). Recently, Gutiérrez-Escritano and colleagues (2012) discovered that $BCR1$, master regulator of conventional biofilm formation in $C. albicans$, is regulated by the main effector of the regulation of Ace2 and morphogenesis (RAM) signaling pathway, Cbk1p-Mob2p complex (Fig. 2). Therefore, it is tempting to assume that PGE$_2$, and other lipid metabolites, can stimulate biofilm formation via these two, and possibly other regulatory pathways.

![Diagram of morphogenesis regulation](Image)

**Fig. 2.** Putative regulation of morphogenesis in $C. albicans$, with PGE$_2$ as a signaling molecule, by multiple signal transduction pathways. Bcr1 and Cph1 are the key transcriptional regulators of conventional and pheromone-stimulated biofilms respectively. Zap1 negatively regulates matrix development. Kic1 and Cbk1 represent the RAM network. Kinases are coloured in dark blue. Stimulatory (→) and inhibitory (←) effects are indicated by arrows. Solid and broken arrows do not establish whether the effect is strong or weak. Kinases are bolded in blue and transcription factors are texto into rectangles.

### 4. Difficulty in genetic analyses of $C. albicans$

Tackling $C. albicans$ pathobiology presents barriers that negatively impact analysis of the underlying genetic determinants. These include difficulty in assessing drug resistant determinants, virulence factors such as morphogenic transitions (e.g. white-to-opaque and yeast-to-hypha transition) as well as biofilm formation. Although
different phenotypes of *C. albicans* are visible and can be assessed by routine assays, their underlying genetic mechanisms which coordinate varying opportunistic traits of clinical isolates, are difficult to assess. One of the barriers hampering *C. albicans* genetic analysis is its diploid nature which is mostly observed in clinical isolates. To genetically manipulate these strains, two rounds of allelic alterations are required (Jones et al., 2004; Nobel & Johnson, 2005, 2007; Nobel et al., 2010). *Candida albicans* also lacks natural plasmids required for transformation and its genome does not display the traditional codon usage in which the CUG codon encodes a serine residue. This results in the need for codon optimization for heterologous markers to be functional in transformation experiments (Santos & Tuite, 1995). Furthermore, expression and mutagenic studies have demonstrated that the use of selectable, especially auxotrophic markers, negatively impacts virulence in *C. albicans* (Lay et al., 1998; Staab & Sundstrom, 2003; Brand et al., 2004). Moreover wild type strains are potentially resistant to dominant markers such as Geneticin (G418) and Hygromycin, generally used for selection in other yeasts (Griffiths, 1995; Santos & Tuite, 1995). The latter has prompted the development of new selection drugs. Some of these drugs developed up to date include Mycophenolic acid (MPA), an inosine monophosphate dehydrogenase inhibitor, and Nourseothricin (NTC) which inhibits protein synthesis and induces protein misfolding (Köhler et al., 1997; Reuss et al., 2004). To utilize these markers, resistant *C. albicans* strains are genetically engineered by introducing a fragment containing a gene that can produce a protein required for survival in the presence of these compounds.

In the face of the previously mentioned barriers researchers have developed genetic manipulation approaches used for mutant construction. Traditionally, genetic manipulation was achieved randomly via the use of chemical mutagens, transposable elements and UV-enhanced mitotic recombination techniques (Fonzi & Irwin, 1993). Such mutagenic methods often resulted in redundant genetic alterations that are difficult to reverse when identified. Therefore, constructed mutants would usually display vague phenotypic outputs which ultimately complicate interpretation of results. Due to these roadblocks, genetic manipulation efforts have been improved over the years to study pathogenesis and virulence. In addition, a fully sequenced genome (http://www.candidagenome.org/) is made available for the *C. albicans* research community to facilitate genetic studies (Jones et al., 2004). Molecular biology techniques, including protocols for gene disruption, are used in connection with this genome database and available mutant libraries (Homann et al., 2009; Nobel et al., 2010; Lohse et al., 2013; Nobile et al., 2012; Guan et al., 2013; Pérez et al., 2013; Si et al., 2013). Mutant libraries in combination with *in vitro* or *in vivo* infection models are, on the other hand, useful in probing transcriptional networks modulating virulence genes. In this review the emphasis will be placed on the common gene deletion methods routinely used to create *C. albicans* mutant strains. Furthermore, characterization of some of the important genetic determinants in *Candida* biology will also be highlighted.

5. Genetic analyses of *C. albicans*: from traditional to current methods of gene knockout

*Saccharomyces cerevisiae* diverged from *Candida albicans* about 900 million years ago (Pesole et al., 1995; Hedges, 2002). This ancestral relationship might explain
numerous similarities observed between these two yeasts such as the amino acid-sensing complex, SPS (Ssy1p, Pr3p and Ssy5p) (Abdel-Sater et al., 2004, 2006; Biswas et al., 2007; Brega et al., 2004; Wu et al., 2006; Davis et al., 2011), the RAM network (Saputo et al., 2008), several DNA binding proteins (Kadosh & Johnson, 2001; Homann et al., 2009) and the mating type loci (MTLa and MTLa) (Hull et al., 1999). Parallelism of features between C. albicans and S. cerevisiae made the adaptation of biological protocols, routinely used for transformation of S. cerevisiae, simple to allow manipulation of the diploid genome of C. albicans. These include the use of URA3 as an auxotrophic marker in a spontaneous recombination system used to sequentially transform C. albicans isolates (Fonzi & Irwin, 1993). One of the modifiable methods already used in S. cerevisiae, with a possibility of applications in C. albicans, is a counter selectable reporter (CORE) system (Storici et al., 2001; Samaranayake & Hanes, 2011). This system can be modified by introducing dominant selectable markers (e.g. MPA and NTC) currently effective in C. albicans (Storici et al., 2001; Storici and Resnick, 2006). Furthermore, gene expression studies involving the use of C. albicans hypomorphs i.e. alleles with faintly expressed phenotypes as opposed to destroyed/abolished phenotypes, initially applied in S. cerevisiae (Yan et al., 2008), have recently been used to detect intermediate allelic functions in C. albicans (Finkel et al., 2011).

In this review, deletion techniques have been divided into spontaneous (URA blaster and the UAU methods), enzymatically-induced recombination systems (FLP- and CRE-mediated methods) as well as PCR-mediated deletion system, which plays an important role in the construction of spontaneous and enzymatically-induced systems applied in the presence of auxotrophic and/or dominant markers. Induced and/or spontaneous systems imply that a particular system does/does not incorporate any additional recombinase (or recombinase-like) protein to recycle the deletion cassette for a subsequent round of genetic transformation. All of these gene deletion systems leave a scar or short sequence at the genome of C. albicans after the target gene is deleted. Recently, the only scarless system, CRISP, which employs Cas9p nuclease, has been described (Vyas et al., 2015) and will also be discussed in this review.

5.1 Spontaneous recombination systems

5.1.1 URA blaster

The development of the URA blaster technique was a step forward in C. albicans genetic research. This technique was developed from studies involved in the genetic manipulation of C. albicans clinical isolates in efforts to make a strain nutritionally competent for growth on medium lacking uracil (Fonzi & Irwin, 1993). As indicated in Fig. 3, URA blaster entails the deletion of the target gene, using a construct that carries the URA3 gene containing two hisG sequences from Salmonella typhimurium. After an allele of the target locus is deleted, C. albicans isolates, including those carrying the disruption fragment, are plated on uracil-deficient medium for selection. Isolates which lack the deletion fragment cannot synthesize uracil since they also lack URA3 gene essential for the production of orotidine-5’-monophosphate (OMP) decarboxylase (ODCase). The short distance between the hisG sequences allow for spontaneous homologous recombination to occur, leading to subsequent removal of the URA3 gene. This process of counter selecting the
URA3 gene is carried out on medium supplemented with 5-fluoroorotic acid (5-FOA) to screen for isolates with an excised URA3 gene (URA). Consequently, a copy of the hisG sequence is placed at the target locus. Positive isolates are resistant to the toxicity of the metabolic product of 5-FOA, 5-fluorouracil. Since the URA3 marker is spontaneously recycled, a second round of transformation is carried out to delete the second copy of the target gene. After this final transformation step, C. albicans strain will be homozygous for the specific deletion such that one allele carries the hisG sequence and the other carries the complete deletion cassette (Fig. 3A) (Fonzi & Irwin, 1993; Berman & Sudbery, 2002).

The URA blaster method has been employed and documented in a number of published papers aimed at determining the underlying genetic aspects of virulence of C. albicans (Smith et al., 1992; Huh et al., 2001; Inglis & Johnson, 2002). However, other studies have indicated that the auxotrophic marker, URA3, has a negative impact on virulence of auxotrophic strains (Kirsch & Whitney, 1991; Lay et al., 1998). This gene is required for hyphal growth and virulence in C. albicans and it was also discovered that ura3/ura3 mutants do not form biofilms on Spider medium (Lay et al., 1998). In addition, the final transformation step in the URA blaster method does not involve selection using medium containing 5-FOA, increasing the possibility of using URA+ strains that have not undergone recombination. This also suggests that a copy of the URA3 gene is left and could be expressed at the locus of the target gene at insufficient levels. This non-native or ectopic URA3 expression has been reported to greatly decrease C. albicans virulence, complicating interpretation of phenotypes (Lay et al., 1998).

In a study involving the re-evaluation of C. albicans mutant strains constructed using the URA blaster method, in comparison with wild type strains, ODCase activities were determined (Lay et al., 1998). This study revealed a double to 18-fold decline in ODCase activity and that this significant decline results in reduced germ tube formation by constructed mutant strains. This is a clear indication that sufficient URA3 mRNA levels are required, ultimately suggesting that virulence of C. albicans strains partly depends on ODCase expression levels. Therefore, previously detected decreased virulence activities measured by the URA blaster method were also influenced by non-native expression levels of URA3. To further support these arguments, Brand and co-workers (2004) established the relationship between the ODCase activity levels and ectopic expression of URA3 mRNAs which was also discovered in 30% of published mutants. Based on these studies, the validity of virulence results obtained using the URA blaster method should be scrutinized to determine whether phenotypic changes observed are solely the result of missing targets or ectopic expression of the URA3 gene.

Nonetheless, measures were put in place to compensate for the negative impacts of URA3 on virulence. For instance, Candida integration plasmid (Cip) vectors (e.g. Cip10, Cip20 and Cip30), are used to circumvent positional locus effects of the URA3 gene (Murad et al., 2000; Dennison et al., 2005). These vectors can be used to express the URA3 gene at highly expressed loci where optimum ODCase levels can be maintained (e.g. ARG4, ENO1 or RPS1) or restore wild type properties by expressing this gene at the native locus (Lay et al., 1998; Murad et al., 2000; Brand et al., 2004). Using the mouse tail vein infection model, Noble and Johnson (2005) demonstrated that virulence in C. albicans strains is less affected using alternative nutritional makers such as HIS1, LEU2 and ARG4. According to their analysis,
strains auxotrophic for alternative nutritional markers or a combination between the three, are more infective and reliable to use. On the other hand, strains with triple auxotrophies that exclude \texttt{URA3} become defective since their metabolic pathways, especially amino acid metabolism, are compromised. These findings further demonstrate the metabolic impacts presented by nutritional markers in \textit{C. albicans} growth and virulence. However, some studies have used \textit{C. albicans} \texttt{URA3} null mutants without reported complications in assessing virulence mechanisms (Jackson et al., 2007; Balish, 2009).

Other problems associated with the URA blaster technique include chromosomal or genetic defects in \textit{C. albicans} strains emerging from exposure to 5-FOA containing medium, making this step an apparent source of unwanted genetic traits (Wellington et al., 2006). This further warrants the use of independent isolates of a mutant strain for reliable phenotypic tests. In addition, using the same cassette to disrupt the second allele of the target gene is sometimes fruitless. This is because the majority of mutants result from the reintegration of the cassette into the first knockout locus or elsewhere in the genome. In some cases, more than 100 transformants have to be screened to obtain a single homozygous deletion mutant (Noble & Johnson, 2005; Samaranayake & Hanes, 2011).

5.1.2 UAU method

\textit{Candida albicans} homozygosis can also be achieved through mitotic events (e.g. gene exchange between a chromosome pair) using the UAU (Uracil-Arginine-Uracil) method (Enloe et al., 2000). This method results in homozygous gene disruption after a single round of transformation depending partly on genetic cross-over events (Fig. 3B). The UAU method uses a strain, auxotrophic for uracil (\texttt{URA3}) and arginine (\texttt{ARG4}) i.e. double auxotrophic strain (Enloe et al., 2000; Nobile & Mitchell, 2009). The UAU cassette contains flanking sequences that are homologous to sequences flanking the target gene and a wild type copy of the \texttt{ARG4} gene, flanked by 5’ and 3’ portions of the \texttt{URA3} gene. After selection of auxotrophic strains on arginine-deficient media, the Arg’Ura phenotype can be confirmed. Transformed strains will undergo genetic recombination and transfer the deletion cassette to delete the next allele. At this stage, homozygous strains (Arg’Ura/Arg’Ura) are produced that contain the disruption fragment at the target loci. Strains are then allowed to grow on medium without selection pressure to allow for spontaneous recombination between two \texttt{URA3} portions. This is followed by subsequent excision of \texttt{ARG4} leaving behind a copy of \texttt{URA3} at the target locus (Enloe et al., 2000; Berman & Sudbery, 2002; Nobile & Mitchell, 2009). However, like the URA blaster, the UAU technique is prone to ectopic expressions of the \texttt{URA3} gene, necessitating the use of integration vectors or an additional transformation step to restore wild type properties.
Fig. 3. A. URA blaster technique is a spontaneous allelic disruption method that applies the URA3 gene flanked by hisG repeats. B. UAU1 disruption technique results in homozygous mutants in a single transformation step. Transformation of C. albicans auxotrophic strain followed by excision of the ARG4 gene.

5.2 Enzymatically induced recombination systems
5.2.1 URA flipper
One of the interesting developments in C. albicans mutant construction was the use of a site-specific recombination system. In this system, a group of enzymes termed recombinases or integrases, such as cyclic recombinase (CRE) and flipase recombinase (FLP), which have been applied in bacteria, mice and plant genomes, are used (Lyznik et al., 2007; Nern et al., 2011). In C. albicans, the FLP-mediated approach, called the URA flipper, where the FLP expression is modulated by C. albicans secreted aspartyl proteinase promoter (SAP2P), is used (Fig. 4A) (Morrison et al., 1993; Morschhäuser et al., 1999). The principle of this technique is dependent upon the use of a deletion cassette comprising URA3 gene as a selectable marker and an inducible SAP2P-FLP fusion. The cassette is flanked by the FLP recognition target (FRT) sequences which can be up to 350 base pairs (bp) long. After successful integration of the FLP cassette into the genome of C. albicans for allelic disruption, the SAP2 promoter is activated to express FLP on SAP-inducing Yeast Carbon Base medium supplemented with bovine serum albumin. Expression of FLP

20
induces recombination between the FRT sequences which removes the auxotrophic marker, URA3. This whole process allows the recycling of the selectable marker for a second round of genetic deletion (Morschhäuser et al., 1999). After the second round of transformation, the target locus will comprise the 350-bp FRT in-between non-coding regions flanking the deleted gene (Fig. 4A).

The URA flipper method is valuable because the frequency of recombination is increased, since it is induced by the FLP-recombinase enzyme. As a result, the efficiency of marker regeneration is increased and the need for selection of positive transformants on toxic 5-FOA medium is eliminated, avoiding undesirable chromosomal translocations and other genetic defects. An additional advantage of this strategy is that it allows marker substitution, suggesting that dominant markers can also be incorporated (Morschhäuser et al., 1999; Reuss et al., 2004). However, since the final step of this technique leaves behind a 350-bp scar at the locus of the target gene, the expression and future knockout studies (i.e. creation of double or triple deletion mutants) of adjacent genes might be affected (Meier et al., 2010). However, the extent to which C. albicans genome is influenced by placement of FRT or other foreign sequences is not highlighted in literature. Nevertheless, this sequence intervention should be taken into consideration in the context of follow up gene expression studies.

5.2.2 MPA<sup>R</sup> and SAT1 flipper

The use of antibiotics, drugs or dominant selectable markers in C. albicans genetic analysis is appealing to researchers concerned with mutant studies of clinical isolates. These markers are favourable because strains tested are wild-type, lacking any auxotrophies, which suggests that they possess less artificially interrupted metabolic patterns that might influence virulence. Furthermore, dominant markers allow for drug targeting studies which assess resistant strains in clinical settings for development of antifungal therapies (Roemer et al., 2003; Shen et al., 2005). However, since wild type strains are often resistant to commonly used dominant markers (Griffiths, 1995; Santos & Tuite, 1995), this has prompted the development of new selection drugs. One of the commonly used alternative markers is mycophenolic acid (MPA) which potently inhibits de novo synthesis of guanine triphosphate, resulting in decreased DNA and mRNA levels. The affected protein in this pathway is the inosine monophosphate dehydrogenase (Imh3p) which is responsible for the production of xanthosine monophosphate from inosine monophosphate. Consequently, a dominant selectable marker (MPA<sup>R</sup>) for C. albicans strain transformation, conferring resistance to MPA, was developed (Wirsching et al., 2000; Köhler et al., 1997, 2005). This marker is adapted from the URA flipper strategy where FRT recombination sequences are used to flank the FLP-recombinase gene, under the SAP2 promoter, with the MPA<sup>R</sup> marker replacing URA3 (Fig. 4B). Sequential gene disruption of the target gene using MPA<sup>R</sup> flipping technique is similar to the URA flipper. However, this technique also leaves behind a FRT scar at the target locus which may impact on transcription of nearby genes.

Nourseothricin (NTC) is also used as a dominant selectable marker for targeted gene mutagenesis. Introducing the streptothricin acetyl transferase (SAT1) gene into C. albicans cells confers resistance to NTC during selection (Reuss et al., 2004; Shen et al., 2005). In FLP-mediated recyclable marker system, NTC is used with
**FLP** gene but in this case a maltase promoter (**MAL2**) regulates expression of the deletion marker (Reuss et al., 2004). The expression of **FLP**-recombinase is activated by growing cells in medium containing maltose (Reuss et al., 2004; Shen et al., 2005; Kopke et al., 2010). After final integration, the FRT sequences are also placed at the locus of the target gene (Fig. 4C).

**Fig. 4.** Deletion methods based on **FLP**-mediated recombination. A. The URA flipper method is used for deletions in auxotrophic strains. B. URA flipper is modified into the **MPA**R flipp**er** which is used for targeted gene disruption in C. albicans wild-type strains. C. The SAT1 flipper method is similar in principle to the URA and MPA**R** methods. However, more regions have been introduced to optimize this system, such as the **FLP** recombinase gene (**FLP re**) which is now controlled by the maltose promoter, **MAL2P**, and the SAT1 gene, which is the resistant gene required for selection. In addition, the **URA3** terminator is inserted following the marker gene.

### 5.2.3 Cre-loxP flipper

The Cre-loxP system was previously studied in Phage P1 where it was encoded as 34-bp loxP fragments comprising 13-bp inverted repeats separated by a core repeat of 18-bp and a **CRE** protein of 38-kiloDaltons (kD) (Hoess & Abremski, 1985; Abremski et al., 1986). Sauer (1987) experimented with *Saccharomyces cerevisiae* and attempted to insert a gene encoding the **CRE** enzyme into this yeast. In this study, the **CRE** gene was expressed under the **GAL1** promoter and recombination was mediated by **CRE**. In *Candida albicans*, the Cre-loxP system is adapted to utilize the principle of efficient marker recycling, avoiding selection on medium which may produce undesirable genetic defects (Dennison et al., 2005). In this yeast, this system constitutes three components which are: 1) the disruption cassette comprising **ARG4**, **HIS1**, and **URA3** auxotrophic markers, each of which is flanked
by the loxP elements, 2) a cassette which contains an artificial, codon-optimized CRE gene whose expression is regulated by the MET3 promoter, flanked by ARG4 fragments (Care et al., 1999) and 3) three C. albicans integration vectors (Murad et al., 2000). These vectors are constructed for phenotypic restoration where in which mutant strains will comprise the gene corresponding to a deleted locus reintegrated into their genome. These vectors are therefore crucial in circumventing the URA3 gene positional effects.

The application of the Cre-loxP flipper is depicted in Fig. 5A. In two separate transformation steps, alleles of the target gene are disrupted using cassettes carrying two different nutritional markers, HIS1 and ARG4. Following this, isolates are plated on corresponding deficient medium to screen for positive transformants. In a third transformation step, an arg4::MET3-cre-URA3 cassette is integrated into the allele that carries the ARG4 cassette such that resulting deletion strains only carries the MET3 regulated cre-URA3 and ARG4 cassette. This is followed by selection of positive transformants on methionine and cysteine which repress expression of MET3 promoter, and subsequent cultivation of isolates on methionine and cysteine deficient medium to activate the MET3 promoter (Care et al., 1999). MET3P activation results in the expression of the CRE recombinase which will catalyze recombination between the loxP sequences. This recombination will loop out the auxotrophic markers such that only loxP sequences are left in the locus of the target gene. There is no report suggesting that loxP sequence may impact on transcription of nearby genes. However, insertion of these sequences at the target locus still has the potential to result in transcriptional irregularities. In addition, the CRE recombinase has been reported to prematurely catalyze recombination of the loxP-MET3p-cre-loxP cassette in Escherichia Coli cells (Dennison et al, 2005). Therefore, this method can be tedious and time consuming since a third transformation step that reintegrates the MET-cre fragment is needed.

5.2.4 Clox flipper

The Clox flipper was developed to complement the disadvantages observed in the Cre-loxP flipper. The Cre-loxP system undergoes premature recombination because of the sufficient expression of the CRE recombinase in E. coli (Dennison et al., 2005). This adds a burden of an additional yeast transformation step to reintegrate the MET-cre fragment to knock out URA3. The Clox flipper on the other hand can overcome this problem. As depicted in Fig. 5B-C, this deletion cassette is equipped with a TUB2 intron from C. albicans, inserted internally in the CRE gene, which prevents expression of the CRE recombinase in E. coli and a 3' untranslated ADH1 and the CYC1 terminator sequences (Shahana et al., 2014). In addition, what makes the Clox flipper different from previously discussed deletion strategies is that, it can be used both in auxotrophic and wild type (prototrophic) strains, permitting analysis of laboratory and clinical strains. This is possible because, in addition to the known auxotrophic marker, URA3, it incorporates the dominant marker, NAT1, which allows for selection on NTC. Both these cassettes i.e. URA3-Clox and NAT1-Clox, can be used along with previously described auxotrophic deletion cassettes (multi-marker deletion) such as those containing the HIS1 and ARG4 genes or alone (single marker deletion).
In a multi-marker deletion strategy, the first allele is deleted with a cassette containing either HIS1 or ARG4 to produce a HIS+ or ARG+ strain. This is followed by deletion of the second allele with the Clox flipper containing either URA3 or NAT1. In between these deletion steps, transformants are selected on methionine and cysteine synthetic complete (SCm/c) medium for the repression of the MET3P-cre. Before marker regeneration, transformants are then plated on fresh SCm/c medium to remove untransformed yeast cells. In both these approaches, CRE, under the regulation of the Met3p promoter, catalyzes recombination between the loxP elements, allowing marker regeneration. This process is carried out by inoculating isolates in SC broth that lacks methionine and cysteine, which activates MET3P-cre, supplemented with histidine, arginine and uridine and followed by plating on the same medium. In the case where cells transformed with NAT-Clox, transformants are selected on YPD medium supplemented with 200 µg/mL NTC. In all instances i.e. in multi-marker and single marker deletion strategies, PCR can be used to confirm the genotypes of transformants instead of passaging them on 5-FOA, which may induce chromosomal alterations. As seen with previous deletion systems, the Clox flipper also leaves behind sequences at the locus of the target gene.
Fig. 5. Deletion methods based on CRE-mediated recombination. A. 34-bp loxP flanked disruption cassettes comprising different auxotrophic markers and ARG4 flanked cassette comprising MET3P-cre fusion and URA3 selectable marker. Deletion based on the multi-marker (B) and single marker (C) systems of the Clox flipper. The CRE recombinase (CRE re) is interrupted by the TUB2 intron (Int.).

5.2.5 CRISPR system

Vyas and co-workers (2015) have developed a deletion system in C. albicans that comprise a Candida-compatible CAS9 nuclease, an artificial guide RNA (sgRNA) and a protospacer motif (PAM). CAS9 encodes a DNA endonuclease enzyme which plays an important role in Streptococcus pyogenes adaptive immunity where it unwinds and cleaves foreign DNA such as invading bacteriophage or plasmid DNA. This system has recently been highly appreciated for its potential role in genome editing in humans and microorganisms (Carroll, 2013; Cho et al., 2013; Jiang et al., 2013). The clustered regulatory interspersed short palindromic repeats (CRISPR)-associated Cas9 endonuclease system adapted by Vyas and co-workers (2015) also appears to be an efficient deletion system in generating C. albicans mutant strains. In this yeast, this system comprises the codon optimized CAS9 gene fused to the nuclear localization signal, SV40, to its 3’ end and placed under the ENO1 promoter (Vyas et al., 2015). In addition, the sgRNA, required for directing Cas9p to the site of cutting, is placed under the RNA polymerase III promoter, SNR52P. Deletion using the CRISPR system follows the use of two fragments, the Duet and Solo systems. The Duet system comprise of two plasmids, pV1025 and pV1090, which target two loci, ENO1 and RP10, respectively (Fig. 6A). Both these systems result in transformants at different frequencies. The Solo system, which is twice as effective in producing transformants when compared to the Duet system, comprise of one plasmid, pV1093, which targets the ENO1 locus (Fig. 6B).

In a single transformation step, both copies of the target gene or multiple members of a single gene family can be deleted using either the Duet or Solo system (Fig.
6C). After transformation, the Cas9 nuclease is directed to the cleavage site by sgRNA. Here the nuclease will cleave sequences that hybridize to the 20-bp protospacer from sgRNA that are preceded by the mutated PAM which prevents repeated cleavage following integration into the genome (Vyas et al., 2015). In addition, this system is also effective in studying essential genes when a regulatable promoter such as MAL2P is introduced (Vyas et al., 2015).

The CRISPR system overcomes many of the problems associated with genetically analyzing *C. albicans* such as having to conduct multiple transformation rounds in order to obtain a single homozygous deletion mutant. But perhaps the most important features that truly differentiate this system from previously discussed systems is the possibility of obtaining mutant strains without selection and leaving behind sequences at the locus of the target gene. In addition, this system also allows researchers to discriminate essential genes from those that are not, using functional analysis (Vyas et al., 2015). Moreover, guide sequences required for Cas9p to cleave target sequences have been identified using the most recent diploid assembly of *C. albicans*. Around 98% of genes can be targeted by 91% of guides which target cleavage at both alleles, while 9.9% guides target one of two alleles. In addition, most of these guides are located around 15-bp of the target site (Vyas et al., 2015), making guiding as accurate as possible.

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**Fig. 6.** *Candida* CRISPR gene inactivation system. A. The Duet system comprises of *ENO1* and *RP10* targeted constructs. B. The Solo system comprises of a single construct. C. Cas9p targeted gene inactivation where the double stranded target DNA is scanned for ~20-bp spacer.
Table 1.
Deletion methods used for *C. albicans* genetic analysis of virulence determinants.

<table>
<thead>
<tr>
<th>METHOD</th>
<th>CASSETTE(S)</th>
<th>MARKER(S) USED</th>
<th>SELECTION</th>
<th>MARKER REGENERATION</th>
<th>PARENTAL STRAIN</th>
<th>CARRIER PLAMID</th>
<th>REFERENCE</th>
</tr>
</thead>
<tbody>
<tr>
<td>URA blaster</td>
<td>hisG-URA3-hisG</td>
<td>Auxotrophic</td>
<td>Uracil-deficient</td>
<td>Spontaneous</td>
<td>CAI4</td>
<td>pURA3Δ::A</td>
<td>Fonzi &amp; Irwin, 1993</td>
</tr>
<tr>
<td>PCR-based URA</td>
<td>URA3-dpi200</td>
<td>Auxotrophic</td>
<td>Uracil-deficient</td>
<td>Spontaneous</td>
<td>RM1000, BWP17</td>
<td>pDDB57</td>
<td>Wilson et al., 2000</td>
</tr>
<tr>
<td>blaster</td>
<td>ura3-ARG4-ura3</td>
<td>Auxotrophic</td>
<td>Arginine-deficient</td>
<td>Spontaneous</td>
<td>CAI4</td>
<td>pBME101</td>
<td>Enloe et al., 2000</td>
</tr>
<tr>
<td>URA flipper</td>
<td>FRT-SAP2P-FLP-URA3-FRT</td>
<td>Auxotrophic</td>
<td>Uracil-deficient</td>
<td>Induced enzymatically</td>
<td>CAI4</td>
<td>pSFU</td>
<td>Morschhäuser et al., 1999</td>
</tr>
<tr>
<td>MPA&lt;sup&gt;®&lt;/sup&gt;</td>
<td>FRT-SAP2P-FLP-MAP&lt;sup&gt;®&lt;/sup&gt;-FRT</td>
<td>Dominant</td>
<td>Mycophenolic acid</td>
<td>Induced enzymatically</td>
<td>Any strain</td>
<td>pSF11</td>
<td>Wirsching et al., 2000</td>
</tr>
<tr>
<td>SAT1 flipper</td>
<td>FRT-SAP2P-FLP-SAT1-FRT</td>
<td>Dominant</td>
<td>Nourseothricin</td>
<td>Induced enzymatically</td>
<td>Any strain</td>
<td>pA83</td>
<td>Reuss et al., 2004</td>
</tr>
<tr>
<td>Cre-loxP flipper</td>
<td>loxP-HIS1-loxP:loxP-ARG4-loxP:arg4-MET3P-cre-URA3-arg4</td>
<td>Auxotrophic</td>
<td>Arginine- histidine- and uracil deficient medium</td>
<td>Induced enzymatically</td>
<td>BWP17</td>
<td>pMAC</td>
<td>Dennison et al., 2005</td>
</tr>
<tr>
<td>Clox flipper</td>
<td>URA3-Clox (loxP-URA3-MET3P-cre-loxP) and NAT1-Clox (loxP-NAT1-MET3P-cre-loxP)</td>
<td>Auxotrophic and/or dominant</td>
<td>Uracil-deficient and nourseothricin</td>
<td>Induced enzymatically</td>
<td>RM1000 (URA3-Clox) and any strain (NAT1-Clox)</td>
<td>pLUCL2 (URA3-Clox) and pLNMC (NAT1-Clox)</td>
<td>Shahana et al., 2014</td>
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<td>ENO1P-CaCas9-FRT:FLP-NAT&lt;sup&gt;®&lt;/sup&gt;-FRT-ENO1, SNR52P-sgRNA-NAT&lt;sup&gt;®&lt;/sup&gt;-RP10 and ENO1P-CaCas9-NAT&lt;sup&gt;®&lt;/sup&gt;-SNR52P-sgRNA-ENO1P</td>
<td>Dominant</td>
<td>Nourseothricin</td>
<td>Induced enzymatically (pV1025)</td>
<td>Any strain</td>
<td>pV1025, pV1090 (Duet) and pV1093 (Solo)</td>
<td>Vyas et al., 2015</td>
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5.3 Designing the deletion fragment – the role of PCR in gene disruption

Polymerase chain reaction (PCR) is an indispensable method used in many areas of molecular biology. As a result, it has found applications in gene knock-out studies where it is used to create deletion fragments (Berman & Sudbery, 2002; Noble & Johnson, 2005; Noble et al., 2010), to introduce point mutations (Nollau & Christoph, 1997) or to perform site directed mutagenesis (Liu & Naismith, 2008). In PCR-based deletions, depicted in Fig. 7A, the marker genes [including ~500-1000-bp up- and downstream the target gene (flanking regions)] are cloned into a vector. Following this, is the separation of the target gene from flanking regions using inverse PCR and inverse primers as well cloning of the marker gene in between flanking regions. The complete deletion fragment is then synthesized with another PCR round using gene-specific primers from the vector DNA (Noble & Johnson, 2005). This linear fragment, which comprises guide flanking sequences, can then be used to transform \textit{C. albicans} and produce desired mutant strains. Berman and Sudbery (2002) described a different approach which also makes use of PCR to generate deletion fragments flanked by ~70-bp guide sequences (Fig. 7B). In this system, primers which contain ~70 sequences (at the ends) that are homologous to the gene to be deleted are designed. These primers are then used to amplify the marker from the vector DNA, within which their cloned, resulting in a deletion fragment with ~70-bp sequences homologous to the target gene (Fig. 7B). This fragment can then be used to transform \textit{C. albicans} to produce desired deletion mutants.

![Diagram of PCR in gene disruption](image)

**Fig. 7.** The role of PCR in designing deletion fragments. A. PCR-mediated mutagenesis following cloning of the marker in between flanking sequences of the target gene previously cloned in the multiple cloning site (MCS) of the plasmid DNA. B. PCR-mediated mutagenesis following amplification of the marker gene using primers with ~70-bp homology to flanking target gene sequences.
The PCR-mediated mutagenesis is especially advantageous since any type of marker, auxotrophic or dominant, and different promoter and terminator regions can be incorporated in the deletion cassette. This is possible when primers that align to desired regions to provide expected products are designed. As a result, the PCR method has been very useful in modifying and optimizing deletion cassettes for *C. albicans* genetic research (Morschhäuser et al., 1999; Wirsching et al., 2000; Roemer et al., 2003; Reuss et al., 2004; Dennison et al., 2005; Shen et al., 2005; Shahana et al., 2014). Furthermore, the PCR method permits the use of marker genes isolated from non-*albicans* Candida species (e.g. *C. maltosa* LEU2 and *C. dubliniensis* HIS1), an aspect which has been shown to improve the effectiveness of gene disruption (Noble & Johnson, 2005). In addition, PCR procedures can be applied for most strategies in the designing of the deletion cassettes and can further be used for confirmation of genomic insertions into the host of interest. In fact, the spontaneous and induction-based protocols discussed in previous sections are facilitated by PCR for amplification of the target regions including their homologous sequences which are required for the integration of the deletion construct, as well as screening of positive colonies generated after transformation. If auxotrophic genes are used in PCR-mediated methods, integration vectors can be used for reintegation of the target genes to achieve complete accountability of the resulting phenotypes. Noble & Johnson (2005) incorporated a strain that is auxotrophic for three markers. Two of these markers are used for sequential disruption while one marker is used for the reintegration of the gene corresponding to the deleted locus. However, in the case of wild type strains where dominant selectable markers are used, the need for a third round of transformation is eliminated since these markers do not impact on the growth of the host (Reuss et al., 2004; Shen et al., 2005). This is, however, substituted by complementation studies, which also largely incorporate the PCR method, to restore the function of the lost or gained phenotype (Nobile & Johnson, 2007).

### 6. In vitro characterization of genetic determinants involved in virulence

Making *C. albicans* deletion mutants is the first step in fully assigning roles to a gene or several genes under study or to further explore the role of known genes. After construction, mutants are subjected to varying environmental conditions which bear a resemblance to those found in the host in order to observe phenotypes that are negatively (reduced) or positively affected (enhanced) (Homann et al., 2009). This section will focus on ways of characterizing some of the main genetic determinants in *C. albicans* that govern virulence.

#### 6.1 Characterization of genetic determinants involved in yeast-hyphal switch

Characterizing genes involved in morphogenesis serves an important role in understanding how *C. albicans* can procure and maintain commensal and pathogenic traits in the host (Pande et al., 2013; Pérez et al., 2013; Kavhanaugh et al., 2014). Morphogenesis in this section can be described as the development of yeast cells into other forms such as hyphae, opaque cells or biofilms and also be described in the context of different colony structures (e.g. wrinkled, smooth, filamentous and/or invasive) (Slutsky et al., 1985; Soll, 1992; Homann et al., 2009; Honigberg, 2011). Characterization of genetic determinants involved in phenotypic
switching can be accomplished by inducing a phenotypic switch from, for instance, yeast to hyphal cells by treating the test strains (i.e. wild type and mutant strains) with hyphal-inducing compounds, referred to as environmental cues or signals. These signals are normally characteristic of those maintained in the host micro-environments. Huang (2012) divided environmental signals based on the pathways they induce or repress and regarded them as positive and negative regulators. Positive regulators are transcription factors that bind promoters of hyphal specific genes, after being activated by an environmental signal, and activate these genes to promote hyphal formation. Likewise, negative regulators bind promoter regions of genes that repress hyphal development. These are the downstream components of the Cdc42/Cdc24 (GDP-GTP exchange factors), Gpa2 (α subunit of G protein involved in cAMP-mediated glucose morphogenesis), Ras1 (GTPase involved in starvation-induced morphogenesis), Ssk2 (regulator of Hog1p kinase activation) and Rim8 (pH-induced morphogenesis) signaling pathways. Mutants of these signaling pathways show defectiveness in hyphal production because hyphal specific genes are not expressed at sufficient levels (Nagahashi et al., 1998; Davis et al., 2000; Sanchez-Martínez & Pérez-Martín, 2002; Bassiliana et al., 2003, 2005; Maidan et al., 2005a, b). Also, mutants disrupted for the downstream transcription factors and the genes they regulate show defective hyphal production while those disrupted for transcription factors required for hyphal repression are notably hyper filamentous. These are some of the key genetic determinants required for high frequency phenotypic switching in C. albicans and have been characterized using a set of environmental conditions (Biswas et al., 2007; Huang, 2012).

Genetic determinants involved in hyphal development are normally characterized using germ tube or yeast-hyphal assays. These assays are conducted using hyphal induction media such as Lee’s [supplemented with N-acetyl glucosamine (GlcNAc), glucose or mannitol] and Spider medium as well as yeast peptone dextrose (YPD) supplemented with 10-20% serum (Sudbery, 2011; Lu et al., 2011). Germ tubes are usually the first structures to be formed during hyphal development and these can be counted after two to six hours of incubation at 30 °C and/or 37 °C. Ells and co-workers (2011) were able to induce germ tube production within four hours using a fatty acid metabolite, prostaglandin E2 (PGE2), which was supplemented in RPMI-1640 medium. One of the first reports to demonstrate that PGE2 could also induce yeast to hyphal switch was by Kalo-Klein and Witkin (1990) where they reported an increase in cAMP levels in cells treated with PGE2, which possibly drove formation of germ tubes from the yeast phase. Inducing hyphal formation in a liquid medium supplemented with signals such as serum, GlcNAc or PGE2 at 37 °C is described as short term, because it is carried out only within four to six hours (Sudbery, 2011). This type of assays trigger a quick response of cells towards available nutrients and allow them to assemble secretary vesicles required for polarized growth, typical of hyphal development in C. albicans (Sudbery, 2011). Long term hyphal production is measured on solid surfaces after incubation time of approximately up to seven days (Sudbery, 2011). However, incubation time may differ among C. albicans strains. Hyphal production on solid surfaces is seen as the ability of a single or group of colonies forming a unit to produce filaments on their borders which can lightly or deeply invade the agar surface (Homann et al., 2009; Sudbery, 2011). Yeast-hyphal transition can therefore be assayed using short or long term techniques or both depending on the research question. The long term agar cultivation is also important, not only to determine hyphal growth, but also to determine the invasive
phenotype of the hyphal structures. The invasive phenotype can be described as the ability of mature colonies to invade the agar surface (Homann et al., 2009; Sudbery, 2011). This phenotype is determined after incubation of colonies on rich medium, such as yeast malt extract (YM) or YPD, followed by washing off of surface colonies to observe persistence of these colonies to invade the agar. A more vigorous and quantitative approach can also be used. In this approach colonies are spotted in a checker board pattern in order to minimize proximity effects and analyzed before and after washing off surface colonies, using the gel documentation system, originally invented to measure DNA quantity inside the agarose gel (Zupan & Raspor, 2008; Ells et al., 2014).

6.2 Characterization of biofilm regulation

*Candida albicans* is capable of forming biofilms, comprised of pseudo- and true-hyphae, yeast cells and extracellular matrix which hold these cellular forms together. Biofilm formation is the leading cause of hospital acquired infections as they are able to attach to medical instruments, normally inserted in vital organs of patients undergoing critical medical treatment (Fox & Nobile, 2012; Fox et al., 2014). The main regulator of biofilm formation is Bcr1p (Biofilm and cell wall regulator) and was first characterized by Nobile and Mitchell (2005). The *bcr/bcr1* mutant fails to form proper biofilms, an indication that Bcr1p is required for biofilm formation while ectopic expression of this protein in a *bcr1/bcr1* mutant reverse the defect (Nobile & Mitchell, 2005). Interestingly, the *bcr1/bcr1* mutant is able to form hyphae but these are functionally defective (Nobile & Mitchell, 2005), an indication that Bcr1p is not required for hyphal production but specifically required for biofilm production. Bcr1p also plays an important role in up-regulating genes required for cell-cell and cell-surface interactions. However, Bcr1p is not the sole regulator of biofilm formation. In fact, biofilm regulatory network, like many other regulatory networks involved in controlling *C. albicans* phenotypes, is interconnected and complex than anticipated (Nobile et al., 2012). Using Chromatin Immuno-precipitation, Nobile and co-workers (2012) mapped a regulatory circuit that fine tunes biofilm development through five additional key regulators which were previously only implicated in the process of biofilm development (Nobile & Mitchell, 2005). During characterization of this intertwined circuit, key regulator mutants *bcr1/bcr1*, *tec1/tec1*, *efg1/efg1*, *ndt80/ndt80*, *rob1/rob1*, and *brg1/brg1* failed to form robust biofilms on the catheter and denture infection models (Nobile et al., 2012).

Biofilms can be developed in commercially available media, such as Roswell Park Memorial Institute (RPMI-1640, HiMedia Laboratories) medium. RPMI-1640 is the ideal medium because it is characteristic of the host physiological conditions and was initially developed to culture human cell lines. *Candida albicans* cells incubated over night in YM or YPD agar or broth are starved at 30 °C for 48 hours in synthetic media supplemented with 1% glucose. These cells can then be washed, counted and diluted into Petri dishes containing RPMI-1640 and incubated for another 48 hours at 37 °C to form biofilms (Ells et al., 2011). Alternatively, fresh cells of the test strains are incubated for up to three days at 37 °C in Spider medium on silicone and stained with concanavalin A Alexa Fluor 594 (Nobile & Mitchell, 2005). Biofilms from the test strains can then be characterized in terms of their morphology and architecture using fluorescent microscopy, including confocal laser scanning. Nobile and Mitchell (2005) have applied this technique to show the impact that Bcr1p has
on the architecture of biofilms in *C. albicans* which translates into the robustness of the biofilms to attach to silicone.

6.3 Characterization of white-opaque switching

*Candida albicans* can also take up another form of high frequency switching, called white-opaque switching. This system has been largely characterized by many research groups, in part, because it is a unique switching system that produces mating competent opaque cells. White-opaque switching was first observed in strain WO-1, isolated from a bone marrow transplant patient (Slutsky et al., 1987). Cells in the white state are relatively round and their colonies appear dome-shaped and white on phloxine B supplemented plates (Soll, 1997). Cells in the opaque state are large and elongated and their colonies are flat and appear dark pink on phloxine B plates (Soll, 1997, 2014). White cells express white-specific genes, while opaque cells express opaque-specific genes (Soll, 1997) and also follow distinct programs of filamentation (Si et al., 2013). In addition, it is accepted that Efg1p is the main regulator of white cell filamentation (Biswas et al., 2007; Huang et al., 2012) while Bcr1p, the main biofilm regulator, also regulates opaque cell filamentation (Guan et al., 2013). Both white and opaque cell types are heritable over a number of generations. White-opaque transition is modulated by the mating type locus (*MTL*) while formed opaque cells are mating competent (Miller & Johnson, 2002). Therefore, the *MTL* regulon plays a dual role in *C. albicans* by regulating white-opaque switching and mating (Hull & Johnson, 1999; Miller & Johnson, 2002). During this regulation, the a1 and a2 proteins are encoded by the *MTL* locus and form a complex that repress Wor1p, resulting in the normal white state observed in most clinical isolates (Huang et al., 2006; Zordan et al., 2006, 2007). Therefore, strains that are capable of switching between the two states are expected to be homozygous at the *MTL* locus (Lockhart et al., 2002). However, the reverse is not always true as there is no evidence suggesting that all *MTL* homozygous strains are capable of switching between the two states (Lockhart et al., 2002). Interestingly, *N*-acetylglucosamine and 5% CO2 can induce switching in some natural isolates of *C. albicans* heterozygous at the *MTL* locus, however, such strains cannot mate (Xie et al., 2013).

Recently, Pande and co-workers (2013) characterized a phenotype which they referred to as the GUT (gastrointestinally induced transition) phenotype. GUT cells can only be induced in the mammalian gut and as a result, their metabolism is synchronized for the conditions encountered in this location (Pande et al., 2013). GUT cells also bear a resemblance to opaque cells except that they lack pimples on their cell walls. Interestingly, GUT cells also express levels of Wor1p, refuting the argument that this protein only drives white to opaque but also white to GUT cell formation. Tao and co-workers (2014b) observed a phenotype on the rich medium, YPD, which is distinct from the traditional white and opaque, as well as the GUT cells of *C. albicans* and refer to this as the grey phenotype. The distinction in the grey cells from white and opaque cells was observed in their mating efficiency, which is higher than the white cells but lower than the opaque cells. In addition, the key regulators, Wor1p and Efg1p, are possibly operating co-ordinately in the white-grey-opaque phenotypic switching since the wor1/wor1 efg1/efg1 double mutant could only form grey cells. Kavanaugh and co-workers (2014) have recently discovered a phenotype which is only induced by heavily glycosylated proteins
associated with the epithelial lining of mammals called mucins. The mucin-induced phenotype is distinct from the previously described phenotypes in that mucin-exposed cells cannot form colonies characteristic of opaque colonies and mating structures under mating conditions. In addition, mucin-exposed cells develop independent of Wor1p expression.

Characterization of white-opaque switching is normally based on switching frequencies described as average proportion of colonies displaying opaque sectors or alternating phenotypes, when compared to the typical white colonies, plus the standard deviation (Tong et al., 2014). Colonies can be counted after incubation of the test strains at 25 °C, a temperature known to increase Wor1p expression levels, for 5 to 10 days. In addition, colonies can then be analyzed microscopically for size and surface pimples. In special cases such as when a new cell type is described, global gene expression profiles using cDNA microarrays or RNA sequencing can be performed to determine what the new cell type truly represents (Pande et al., 2013; Tao et al., 2014b). Since white-opaque switching is tightly linked with mating (Hull & Johnson, 1999; Miller & Johnson., 2002; Lohse & Johnson, 2009), mating assays are conducted to dissect mating efficiencies of the different cell types (Tong et al., 2014). Combined, all these techniques can differentiate between varying cell types of C. albicans. Characterization of the white-grey-opaque and GUT or mucin phenotypes (Pande et al., 2013; Kavanaugh et al., 2014; Tao et al., 2014b) lays down a foundation for evaluating C. albicans for more forms that it employs while thriving in host for nutrients. These phenotypic forms further suggest that C. albicans maintains diverse genetic programs that can be activated in specific host niches.

6.4 Characterization of genetic determinants involved in drug resistance

*Candida albicans* causes life-threatening infections in immunocompromised persons, leading to an increasing need to develop broad spectrum fungicidal drugs. However, prolonged use of antifungal drugs by affected patients results in *C. albicans* strains becoming less susceptible to these drugs (Dawson et al., 2011). Fluconazole (FLC), a triazole that specifically targets Erg11p, a lanosterol 14(α)-demethylase, is the most prescribed antifungal drug in patients with fungal infections. However, certain clinical isolates of *C. albicans* developed resistant to this drug due to mutations in the *ERG11* gene which is the sole target of FLC (White et al., 1998, 2002; Xiang et al., 2013).

Genetic mutations are highly implicated in the resistance of antifungal drugs such as FLC. Mutations in the *ERG11* gene, known to alter FLC susceptibility, have been identified in *C. albicans* clinical isolates (White et al., 1998, 2002; Xiang et al., 2013). These mutations can range from point to missense mutations and can compromise binding of *ERG11* to azole drugs. Overexpression of *Candida* drug resistance genes, *CDR1* and *CDR2*, encoding multidrug efflux (ABC) transporters, and *MDR1*, a gene for major facilitator class transporters has been shown to decrease intracellular azole concentrations in resistant *C. albicans* isolates (Sanglard et al., 1995, 1997, 1998, 2003), suggesting that these proteins are capable of exporting the drug out of the cell. Exporting the drugs out of the cell also means offloading them from Erg11p, further decreasing susceptibility and increasing resistance in clinical isolates. On the other hand, *TAC1*, transcription activator of Cdr1p and Cdr2p (Coste et al., 2004) exposes a possible link between *MTL* homozygosis among *C. albicans*
isolates and azole resistance (Rustad et al., 2002) since it is located near the MTL locus. In extension to that, Pujol and co-workers (2003) demonstrate that this might be a poor link since their findings suggest that natural MTL homozygous strains do not always acquire an intrinsic or natural resistance to azole drugs. In addition, gain-of-function mutations in the TAC1 gene sequence result in high activity of Tac1p which enhances transcription of CDR1 and CDR2 (Coste et al., 2004). Mutations in the MRR1 gene, known to regulate MDR1, cause overexpression of Mdr1p, thereby also increasing drug efflux and azole resistance in C. albicans (Coste et al., 2004; Morschhäuser et al., 2007; Morschhäuser, 2010).

Characterization of genes involved in drug resistance is generally performed using the broth microdilution method according to the standards of the Clinical and Laboratory Standards Institute (e.g. CLSI, 2008). The minimum inhibitory concentrations of azole (or polyene) drugs are determined for the test strains in broth medium such as buffered RPMI-1640 medium (Xiang et al., 2013). These concentrations are tailored to determine the extent of resistance or sensitivity of tested isolates towards a particular antifungal drug. Depending on the optimal growth temperature of the yeast, incubation would be conducted accordingly and for a specific period of time. Additionally, genes involved in drug metabolism (e.g. drug targets such as ERG11 or efflux pumps such as CDR1, CDR2 MDR1, FLU1 and SNQ2) can be sequenced in newly isolated resistant strains and compared to the wild-type sequences to detect novel mutations which induce resistance (Morschhäuser, 2010; Xiang et al., 2013). Transcriptional regulators (TRs) which bind promoters of genes coding for proteins playing roles in drug resistance can also be characterized. Morschhäuser and colleagues (2007) characterized MRR1 using genome wide-gene expression (DNA microarrays) by analysing expression levels of this TR during Mdr1p overexpression C. albicans clinical isolates.

6.5 Characterization of the amino acid sensing and signaling: SPS complex

Yeasts possess an amino acid sensing complex, called the SPS complex, which comprise of three genes, SSY1, PTR3 and SSY5 (Biswas et al., 2007; Abel-Sater et al., 2011; Ljungdahl, 2009). In the presence of amino acids, Ssy1p (amino acid sensor), localized in the plasma membrane, is activated and physically interacts with Ssy5p (endoprotease) and Ptr3p (peptide transporter) with its N-terminal domain (Ljungdahl, 2009). Upon activation of these proteins, Ssy5p, via receptor-activated proteolysis, processes the two transcription factors, Stp1p and Stp2p, operating downstream of the amino acid sensing pathway (Ljungdahl, 2009; Pfirrmann et al., 2010). Stp1p and Stp2p, which localize in the nuclear membrane, are required to bind promoters of genes needed for utilization of extracellular proteins (e.g. secreted aspartyl protease) and import of amino acids (amino acid permeases). The Asi complex (Asi1p, Asip2p and Asi3p), also localized in the nucleus, is required to restrict promoter access of Stp1p and Stp2p proteins containing the N-terminal domain (unprocessed) which find their way into the nucleus.

Components of the SPS signaling have been characterized in a number of ways. For instance, the upstream component, SSY1, was characterized in terms of hyphal formation by subjecting the mutant strains (e.g. heterozygous, homozygous and complemented strain) to YPD containing bovine serum, Lee’s, Spider, synthetic low-ammonium dextrose, or GlcNAc medium (Brega et al., 2004) at 37 °C. Additionally,
amino acid uptake and transport was also assessed by growing strains in medium containing labelled amino acids (Brega et al., 2004; Kim et al., 2006). Davis and co-workers (2011) further characterized the SPS signaling in terms of virulence and demonstrated that Ssy1p, Ssy5p and Stp1 deletion mutants display altered virulence in a Drosophila infection model. These findings are consistent with the fact that ssy1/ssy1 strain is defective in filamentous growth and amino acid uptake, crucial in host invasion and infection (Brega et al., 2004). In serum containing medium, deletion of SSY1 impairs colony wrinkling, normally associated with hyphal production while complementation of this gene restores these phenotypes. Interestingly, the SPS complex has recently been implicated in pH regulation in which activation of Stp2p signals an increase in pH and hyphal morphogenesis (Vylkova & Lorenz, 2014).

6.6 Characterization of the RAM Signaling

Regulation of Ace2p transcription factor and polarized morphogenesis, or RAM, is one of the pathways that are conserved across eukaryotes. It has been well studied in S. cerevisiae where it was shown to regulate transcription of chitin synthase genes and affect agar invasiveness (King & Butler, 1998) as well as genes expressed in the G2 phase involved in mitosis and cell wall metabolism (Doolin et al., 2001). In C. albicans, the RAM network also maintains some of these morphological roles. For instance, the ace2/ace2 strains are defective in cell separation and agar invasion as well as in biofilm development (Kelly et al., 2004). In addition to that, the C. albicans ACE2 mutants were shown to possess reduced expression of cell wall associated genes such as CHT3, SCW11 and DSE1. The RAM network is, therefore, essential for determining cell fate asymmetry and polarized budding in yeast cells (Jansen et al., 2006; Song et al., 2008; Saputo et al., 2012). During the RAM signaling, Cbk1p kinase forms a complex with Mob2p which regulates its activation and localization during mother-daughter cell formation (Colman-Lerner et al., 2001; Weiss et al., 2002; Nelson et al., 2003; Song et al., 2008; Saputo et al., 2012), suggesting that Mob2p is necessary for Cbk1p kinase activity. Hym1p and Sog2p also interact with Cbk1p by forming another complex with Kic1p to properly localize the Cbk1p-Mob2p complex (Bidlingmaier et al., 2001; Nelson et al., 2003). The two complexes, Hym1p-Sog2p and Cbk1p-Mob2p are scaffolded into a single complex by Tao3p and activate several cell integrity transcription factors including Ace2p (Song et al., 2008; Saputo et al., 2012). Cbk1p kinase activity is reduced in cells lacking other components of the RAM network (Jansen et al., 2006), implying that the effector protein, Ace2p, is not turned on in these strains. Jansen and colleagues (2006) observed reduced levels of phosphorylation at the T-loop kinase domain in the hym1/hym1 and kic1/kic1 deletion strains and undetectable levels in the mob2/mob2 and tao3/tao3 deletion strains. However, Cbk1p kinase activity is not affected in the ace2/ace2 mutant, suggesting that Cbk1p is the terminal kinase or main effector while Ace2p is an endpoint effector in the RAM signaling. In addition, Cbk1p and Ace2p nuclear localization requires phosphorylation at the CT-motif of the Cbk1p kinase domain, suggesting that this phosphorylation is required to maintain the Cbk1p-Ace2p complex (Saputo et al., 2008).
Characterization of the RAM signaling components is also based on the routine tests such as colony and cellular morphology on different media, and hyphal formation assays previously described. Cell separation and invasion properties can be analyzed using chitin staining assays with Calcofluor white and agar invasion experiments respectively (Kelly et al., 2004). In the genome of *C. albicans*, CBK1 contains putative targets among transcriptional regulators associated with biofilm development such as Ace2p, Bcr1p, Nrg1p and Zap1p (Kelly et al., 2004; Nobile & Mitchell, 2005; Nobile et al., 2006a; Nobile et al., 2009; Uppuluri et al., 2010). Since the Ace2 gene plays a crucial role in biofilm formation (Kelly et al., 2004; Gutiérrez-Escribano et al., 2012), biofilm assays, described in section 6.2, can also be included in the characterization of the RAM signaling. In addition to Ace2p mentioned above, several mutants of the RAM network such as kic1/kic1, tao3/tao3, and mob2/mob2 which display defects in adhesion and biofilm formation can be characterized much in the same way as Ace2p. Bcr1p, main regulator of biofilms, contains two Cbk1p putative sites at T191 and S556 required for adherence and biofilm formation, suggesting that phosphoregulation of Bcr1p by Cbk1p at these putative sites is required for expression of Bcr1p-dependent genes such as ALS3 and ALS1 (Gutiérrez-Escribano et al., 2012). Moreover, Cbk1p-Bcr1p phosphoregulation is also crucial for virulence in *C. albicans*. Therefore, adherence, phosphorylation and virulence assays (infection studies) should be included in the characterization of genetic determinants associated with components of the RAM network.

6.7 Characterization of stress response signaling

*Candida albicans*, like many other fungi, reacts to varying stress conditions and activates stress coping mechanisms under these conditions. Stress response can be induced by factors such as increased temperatures and salt concentrations (Brown et al., 2014). *Candida albicans* heat-shock proteins (HSPs) respond to high temperatures and are activated during this period for thermal regulation (Nicholls et al., 2009). These proteins are chaperones and are responsible for refolding of denatured proteins following exposure to high temperatures (Nicholls et al., 2009). Hsf1p, the main thermal regulator (Leach et al., 2012a), was initially identified in *S. cerevisiae* where it maintains the role of thermal regulation. This protein is conserved across many members of the Ascomycete clade and it plays a vital role in cell viability (Brown et al., 2014). Hsf1p is phosphorylated in response to heat-shock and induce transcription of HSP genes required for growth at high temperatures via heat-shock elements (Nicholls et al., 2009; Leach & Cowen, 2013; Brown et al., 2010, 2014). Following transcription of HSP genes, proteins that are denatured during exposure to high temperatures are either refolded or marked for degradation and this generally allows the pathogen to adapt to thermal stress (Brown et al., 2014).

In the presence of high salt concentrations such as NaCl or KCl, *C. albicans* undergoes osmotic and cationic stress. These stresses cause subsequent loss of water which also affects cell size and turgor pressure. These events activate the high osmolarity glycerol (HOG) signaling which is characterized by the accumulation of the hog1p kinase (Hohmann, 2002; Brown et al., 2014). The hog1p kinase will phosphorylate transcription factors required for the expression of stress related genes such as those involved in glycerol biosynthesis (Hohmann et al., 2002; Brown...
et al., 2014). Glycerol accumulation restores turgor pressure and allows *C. albicans* to adapt to osmotic and cationic shock. In addition, the HOG pathway is also required to colonize the mammalian gastrointestinal (GI) tract. Hog1 deletion mutant shows significant sensitivity towards bile salts and reduced attachment in the GI tract of a mouse (Prieto et al., 2014).

Other stress inducing conditions include antifungal agents (e.g. caspofungin) and chemicals such as Calcofluor White and Congo Red as well as reactive oxygen and nitrogen species (ROS & RNS) (Brown et al., 2014). Caspofungin, Calcofluor White and Congo Red are the common *C. albicans* cell wall stressors *in vitro* (Homann et al., 2009; Brown et al., 2014). These agents act by interrupting β-glucan (Caspofungin and Congo Red) and chitin biosynthesis and assembly (Calcofluor White) (Brown et al., 2014). These *in vitro* cell wall stress signals closely mimic those encountered by the pathogen as it interacts with the host. In *S. cerevisiae*, the cell wall integrity signaling or protein C kinase pathway regulates expression of genes playing roles in cell wall metabolism (Hohmann et al., 2002). Mutants defective in this pathway are resistant to Calcofluor White. Likewise, this pathway is conserved in *C. albicans* and contributes to cell wall stress resistance (Brown et al., 2014). The HOG pathway is also involved in the resistance to ROS and RNS. During exposure to ROS, *C. albicans* AP-1-like transcription factor, Cap1p, and a response regulator Skn7p, are activated. Hog1-independent accumulation of Cap1p inside the nucleus is triggered by oxidation at its redox-sensitive cystein residue at the C-terminal end (Brown et al., 2014). Cap1p then activates transcription of genes involved in oxidative stress detoxification such as catalases and superoxide dismutase, glutathione synthesis, redox homeostasis and oxidative damage repair. Cap1p is also implicated in the regulation of multidrug resistance. The *S. cerevisiae* Cap1p (Yap1p) regulates expression of genes involved in oxidative stress and major facilitator as well as ABC transporter superfamilies (Morschhäuser, 2010). *Candida albicans* also experiences nitrosative stress when subjected to RNS. Reactive nitrogen species include nitric oxide which exert fungistatic effects, to which *C. albicans* responds by activating stress related enzymes such as catalase (Cat1p), glutathione-conjugating and -modifying enzymes, and NADPH oxidoreductases and dehydrogenases (Brown et al., 2014).

Components of the stress response pathway can be characterized based on the stress conditions they are alleged to be involved in. For instance, genes expressing heat shock proteins, such as those controlled by Hsf1p, can be knocked out and resultant mutants can be subjected to high temperatures. Sensitivities to thermal shifts can then be recorded at 25 °C, 30 °C, 37 °C and 42 °C and be ascribed to responses due to deleted genes (Leach et al., 2011, 2012a). In terms of characterizing transcriptional regulators (TRs), mutants can be analyzed using genome-wide gene expression to uncover genes whose expression is affected in the TR mutant (Nicholls et al., 2009). Alternatively, genes upregulated or over expressed after exposure to heat shock can also be detected using genome-wide gene expression studies (Enjalbert et al., 2003, 2009; Nicholls et al., 2009). Genetic determinants controlling osmotic stress can be analyzed by examining sensitivity of mutants towards high salt or sugar content. Fresh, actively growing cells from mutant strains can be serially diluted and spotted onto YPD agar plates, with up to 1.8 M NaCl and Sucrose or 1 M Sorbitol, to induce osmotic stress which activates the HOG pathway (Albertyn et al., 1994; Homann et al., 2009). Sucrose is known to enhance glycerol-3-phosphate dehydrogenase (GPD) mRNA levels during osmotic
stress and the resultant increase in glycerol production in S. cerevisiae (Albertyn et al., 1994). Characterization of osmotic stress genetic determinants can therefore be supplemented with quantification of the glycerol content and expression levels of GDP (Albertyn et al., 1994).

In much the same way as characterization of osmotic stress genetic determinants, mutants can be serially diluted and spotted onto YPD agar plates containing oxidative, nitrosative and cell wall stress inducing compounds and be analyzed after incubation for a specific number of hours or days. Hydrogen peroxide (H$_2$O$_2$) and nitric oxide (NO) can be used to induce oxidative and nitrosative stress genetic determinants respectively (Hromatka et al., 2005; Homann et al., 2009, Kalorit i et al., 2012; Leach et al., 2011, 2012a, b). About 5-6 mM of H$_2$O$_2$, supplemented in YPD agar, is normally enough to induce oxidative stress in test strains (Homann et al., 2009). Nitrosative stress assay is conducted using a buffered medium such as Tris-buffered YPD at pH 7.4 to allow pH-dependent decoupling of NO (and free amide) from Dipropylenetriamide NONOate or DPTA NONOate (Hrabie et al., 1993; Keefer et al., 1996; Kalorati et al., 2012). Mutants can also be spotted on YPD containing Sodium Dodecyl Sulphate (0.04%), Congo Red and Calcofluor White to characterize determinants controlling cell wall stress (Homann et al., 2009, Leach et al., 2011, 2012a, b). Alternatively, strains disrupted for specific genetic determinants can be subjected to corresponding stressors and be monitored in liquid cultures using 96-well microtitter plates. Biological replicates can be incubated stationary at high temperatures (30 °C, 37 °C and 42 °C) for heat stress or at 30 °C for other stresses for 48 hours (Leach et al., 2012a). Following this incubation time, optical density measurements, usually at OD$_{600nm}$, can be carried out for quantification or to score phenotypes.

7. Concluding remarks

This review discussed important mutant construction techniques used in C. albicans genetic analysis, with a focus on their applications and contribution to mutant collection. This review further reveals that mutagenic techniques represent spontaneous and enzymatically induced recombination systems with respect to selection on respective medium. Techniques incorporating a spontaneous recombination system such as the URA3 blaster and the UAU1 method have been developed into induced systems based on the considerations to eradicate targeted locus effects stemming from URA3 non-native or ectopic expression. This type of expression has been directly linked to reduced virulence of C. albicans and perturbed elucidation of important phenotypes (Lay et al., 1998; Staab & Sundstrom, 2003, Brand et al., 2004). Therefore, it is clear that auxotrophic markers are virulence determinants, prompting that mutant construction be supported by complementation studies or the use of additional isolates of the mutant strains generated. Recombinase-based techniques, which apply both auxotrophic and dominant selectable markers, appear to be advantageous over spontaneous recombination techniques. However, their drawback, common with the spontaneous techniques, is the insertion of foreign sequences or scars (e.g. FRT and loxP) into the genome of C. albicans which may possibly affect transcription of adjacent genes or integrate in the cis-Regulatory Elements (CREs) region. However, there are scarce reports in literature showing the effect of genome intervention by these sequences on the CRE region. One method that bypasses this is the CRISPR
system adapted by Vyas and co-workers (2015) for use in *C. albicans* genetic research. This system also presents the possibility of increased transformation efficiency, not observed with other deletion methods, such that selection is eliminated (Vyas et al., 2015). In the CRISPR method, a codon optimized *C. albicans* endonuclease, Cas9p, is guided to the cleavage site by sgRNA where it inactivates the target gene without leaving a scar or foreign sequences behind. In *S. cerevisiae*, the *Aspergillus nidulans* amdSYM has been developed to also overcome the problem of placing foreign short sequences at the locus of the target gene (Solis-Escalante et al., 2013). This system employs a dominant selectable marker, amdSYM, which allows transformed isolates to utilize acetamide as a sole nitrogen source. The amdSYM cassette contains a 40-bp sequence homologous to the upstream region of the target gene which results in the direct repeats in the host upon insertion (Akada et al., 2006; Solis-Escalante et al., 2013). Therefore, this system avoids leaving behind short sequences in the genome of *S. cerevisiae* by incorporating two recombination rounds; one for marker recycling on acetamide and the other for the 40-bp region to remove the short sequence on fluoroacetamide (Solís-Escalante et al., 2013). This system can be optimized for use in *C. albicans* auxotrophic and clinical strains. However, three recombination rounds are inevitable to successfully knockout a specific gene and to remove short sequences.

It is further highlighted in this review that deletion strategies have contributed to mutant collections used to decipher *C. albicans* virulence. Mutant libraries are important in characterizing important genetic determinants representing regulatory circuits governing phenotypic switching and stress response, both of which contribute to virulence (Roemer et al., 2003; Nobile & Mitchell, 2005; Homann et al., 2009; Pérez et al., 2013). More importantly, mutant libraries exploit the roles of sequence-specific DNA binding proteins, whose principal function is to control transcription of functionally related or redundant genes underpinning the biology of pathogenic yeasts. In this review, we have further touched on the characterization of deletion strains representing genetic determinants in the yeast-hyphal (Biswas et al., 2007; Sudbery, 2011; Soll, 2014) and white-opaque switching systems (Huang et al., 2010, 2012; Soll, 2014), biofilm development (Nobile & Mitchell, 2005, 2006), amino acid sensing (SPS signaling) (Ljungdahl, 2009), cell separation, adhesion and polarized growth (RAM signaling) (Song et al., 2008; Saputo et al., 2012) as well as stress response pathways (Hohmann et al., 2002; Leach et al., 2011, 2012a, b; Leach & Cowen, 2013; Brown et al., 2014). It appears that *C. albicans* displays a considerable amount of genome elasticity, evident of the vast phenotypes orchestrated by signaling networks triggered by numerous environmental stimuli. However, studying these phenotypes requires gain or loss of function mutants and at the same time, characterizing these mutants requires conditions that mimic those found in the mammalian host. Therefore, mutant construction and characterization is inevitable to understand roles played by genetic determinants in controlling commensalism (Pande et al., 2013; Pérez et al., 2013) and pathogenicity (Pérez & Johnson, 2013) in a mammalian host and to make an informed decision during drug development. Furthermore, mutant collections should also be considered for applications in the emerging research fields such as the analysis of eicosanoid (e.g. PGE₂) biosynthetic pathways in pathogenic yeasts. This will shed light on how these metabolites are synthesized and how they orchestrate morphogenesis in pathogenic yeast and fungi.
8. Proposed research

The pathogenic yeast, *Candida albicans*, can form prostaglandin E\(_2\) (PGE\(_2\)) from host derived arachidonic acid (AA). A study that was conducted to evaluate the effect of AA on the genome wide expression of genes by *C. albicans* NRRL Y-27077 revealed several open reading frames (ORFs) to be significantly upregulated in the presence of AA (Ells, 2011). Further analysis revealed that seven of these ORFs may be involved in morphogenesis as well as biofilm development in *C. albicans*. According to *Candida* Genome Database (http://www.candidagenome.org/), two of these ORFs were found to represent genes coding for uncharacterized proteins. Since *C. albicans* PGE\(_2\) transcriptional response has previously been characterized, this study aims to extend on the characterization of AA transcriptional response by characterizing some of the genes up-regulated during AA conversion. Three genes, two coding for membrane-bound proteins (ABC transport/pheromone exporter protein (Hst6p) and amino acid sensor (Ssy1p)) and one coding for an uncharacterized protein (Orf19.4612p), will be analyzed. Therefore, the aims of this research are structured as follows:

1. Construction of knockout plasmids containing the *SAT1* flipper (*Chapter II*)
2. Construction of deletion mutants of *C. albicans* orfs previously found to be upregulated during growth in the presence of AA (*Chapter II*)
3. Characterization of the deletion mutants in terms of phenotype, stress response, phenotypic switching and production of PGE\(_2\) (*Chapter III, IV and V*)
9. References


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Chapter II – Candida albicans Mutant Construction of Genes Expressed during Exposure to Arachidonic Acid

1. Abstract
Auxotrophic markers used in C. albicans cell biology have limited applications when it comes to analysis of clinical isolates. This is largely because of the requirement to pre-determine an auxotrophic strain before mutant construction can commence. In addition, literature demonstrates that these markers are a source of problems in the interpretation of virulent phenotypes since they play a role in virulence. To combat these problems, the SAT1 flipper recombination system for the construction of C. albicans null mutants from wild type clinical strains was designed. This system relies on the selection of positive transformants on the drug NTC, given that they retain the resistant gene, Streptomycin acetyltransferase1 (SAT1). It is also based on marker regeneration technology using the flipase (FLP), whose expression is activated by growing cells on maltose medium to activate the regulatable maltose promoter. This promoter activates the FLP to catalyze excision of the deletion cassette located between short FLP recombinase recognition target sequences. We employed this system to transform C. albicans NRRL Y-27077, isolated from a skin lesion. Non-coding sequences flanked by the SphI site, separated by ORFs, were cloned into pGEM®-T Easy and pSMART HC Kan commercial vectors. These were used to flank the SAT1 flipper construct, optimised in pGEM®-T easy with multiple cloning sites, including SphI. Modification of these regions with the SphI restriction site was attained using inverse PCR to obtain compatibility for cloning of the deletion marker, SAT1. During this study, seven knockout vectors were constructed and confirmed by generating restriction fragment length polymorphisms. Out of these, four were used to integrate the loci of HST6, SSY1, ORF19.5574 and ORF19.4612.
2. Introduction

*Candida albicans* is a pleomorphic yeast that thrives in the host niches as single cells, hyphae and biofilms. In the non-pathogenic state, it exists as a commensal strain in the microflora of healthy human beings (Chen et al., 2011; Pande et al., 2013; Pérez et al., 2013; Kavanaugh et al., 2014). However, it may invade important tissues due to impairment of individual's immune response or as a response to a hostile host micro-environment (Felk et al., 2002; Vylkova & Lorenz, 2014). A characteristic feature of this yeast, important in molecular biology and mutant construction, is its diploid nature which makes it challenging to genetically assess important phenotypes contributing to commensalism and pathogenesis. Research indicates that pathogenesis is initiated by activation of genes coding for proteins contributing to virulence. These include those coding for proteins responsible for hyphal formation (e.g. Bcr1p, Cph1p, Efg1p and Tec1p) (Biswas et al., 2007; Nobile et al., 2012; Guan et al., 2013), those participating in the inherent adhesive properties (Als1p, Als3p, Ece1p, Hwp1p and Zap1p) (Nobile et al., 2006a, 2009) and those controlling biofilm development (e.g. Bcr1p, Cph1p, Rob1p and Ndt80p) (Nobile & Mitchell, 2005, 2006; Nobile et al., 2006a, b, 2009, 2012). In addition, there has been an increasing interest in the ability of lipid metabolites such as prostaglandin E\(_2\) (PGE\(_2\)) to induce virulence in *C. albicans* (Kalok-Klein & Witkin, 1990; Ells et al., 2011). However, little is understood about the biosynthetic pathway of PGE\(_2\) and subsequent induction of virulent determinants such as hyphal and biofilm regulation in pathogenic yeasts. So far, it is known that a fatty acid desaturase-like enzyme (Ole2p) and a multicopper oxidase homologue (Fet3p) are involved in the synthesis of PGE\(_2\) in *C. albicans* (Erb-Downward & Noverr, 2007). In *Cryptococcus neoformans* and *Aspergillus fumigates*, a lacasse (Lac1p), a member of the multicopper oxidase family of enzymes, and an oxidase or cytochrome c oxidase, were found to be involved in PGE\(_2\) production (Erb-Downward et al., 2008; Kupfahl et al., 2012).

Genome-wide gene expression using cDNA microarrays and RNA sequencing plays a crucial role in *C. albicans* genetic analysis. This application was made feasible following genome sequencing and development of the *Candida* Genome Database (Jones et al., 2004). From these developments, the assembly of transcription profiles of genes playing roles in yeast to hyphal switching (Nantel et al., 2002) and biofilm formation (Nobile et al., 2012) was made possible amongst others. Moreover, *C. albicans* cDNA microarrays have been used to evaluate expression patterns in order to profile transcription during treatment with antifungal drugs and fatty acids (Backer et al., 2001; Levitin & Whiteway, 2007; Ells, 2011). Microarrays are also useful to study the resistance of biofilms to drugs such as farnesol, a quorum sensing compound that controls morphogenesis and virulence (Cao et al., 2005). Previously, Levitin and Whiteway (2007) used cDNA microarrays for transcriptional analysis of genes differentially expressed in the presence of PGE\(_2\) and discovered that *C. albicans* can sense PGE\(_2\) in its surrounding environment. In our research group, *C. albicans* cDNA microarrays were also used to detect genes differentially expressed during growth in the presence of AA, leading to the production of PGE\(_2\) (Ells, 2011). In this study, it was observed that after 8 h, most differentially expressed genes were down-regulated, whereas after 24 and 48 h there was a shift to more genes being up-regulated. The latter can be supported by the unpublished work of Ells (2011) which demonstrate that PGE\(_2\) production is at its highest after 48 h in the presence
of AA. Therefore, it appears that AA increases gene expression levels and also drives an increase in cellular metabolism, including PGE$_2$ production.

In the current study, we aim to understand whether genes up-regulated during AA metabolism were simply responding to AA, or play a significant role in the pathobiology of *C. albicans*. This chapter will provide an elaborate description of mutant construction of genes coding for two membrane-bound proteins, Hst6p and Ssy1p, and two previously uncharacterized proteins, Orf19.5574p and Orf19.4612p, all of which were significantly up-regulated during treatment with AA (Ells, 2011). In addition, deletion constructs for *SNQ2, HYM1* and *SMT3*, also up-regulated during treatment with AA, were also constructed. However, none of these were successfully deleted.

3. Materials and Methods

3.1 Strains and growth settings

*Candida albicans* NRRL Y-27077 (http://nrrl.ncaur.usda.gov/), isolated from a skin lesion, was used in this study. This strain was maintained at 4 °C on yeast-extract-peptone-dextrose (YPD) agar composed of 10 g/L yeast extract, 20 g/L peptone and 20 g/L glucose, solidified with 20 g/L agar, until further use. A culture of this strain was also preserved at -80 °C in 15% glycerol and, prior to use, was streaked on YPD plates and left to grow at room temperature for 3-10 days, and followed by inoculation in YPD broth for 24 h prior to experimental use.

3.2 Open reading frames and primer design

Sequences of seven ORFs, along with the ~500-bp downstream and upstream flanking sequences, were retrieved from *Candida* genome database (CGD: http://www.candidagenome.org/) (Table 1). Primers used in this study (listed in Table 2) were designed using Primer3 generator (Rozen & Helen, 2000) inside Geneious Pro 5 (Drummond et al., 2011). Three sets of primers were designed; 1) locus- or gene-specific, designed to amplify the flanking sequences, 2) inverse primers, designed to attach the *SphI* restriction site onto the flanking sequences and to delete the target sequences from the flanks contained in the cloning vector DNA using inverse PCR, 3) the *SAT1* flipper primers designed to amplify the deletion cassette and to clone it with multiple restriction sites (including *BglII, EcoRI, NcoI, Nhel* and *SphI*) into a chosen vector. The *SAT1* flipper was provided as a kind gift by Prof. J. Morschhäuser, Institut für Molekulare Infektionsbiologie, Universität Würzburg, Germany. In addition, another primer that targets the *SAT1* gene was designed to confirm integration of *SAT1* flipper into *C. albicans* genome.
### Table 1
Open reading frames retrieved from *Candida* genome database

<table>
<thead>
<tr>
<th>Description*</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Uncharacterized; code for a protein of unknown function; mutants display reduced invasive phenotype</td>
<td><a href="http://www.candidagenome.org/">http://www.candidagenome.org/</a>; Xu et al., 2007; Oh et al., 2010; Singh et al., 2011</td>
</tr>
<tr>
<td>Required for hyphal and biofilm morphogenesis as well as cell wall maintenance</td>
<td>Song et al., 2008; Davis et al., 2011; Sudbery, 2011; Shapiro et al., 2011, Saputo et al., 2012</td>
</tr>
<tr>
<td>Code for potential transporters (e.g. PDR family) involved in multidrug resistance and pheromone transport</td>
<td>Magee et al., 2002; Chang, 2003; Hlavacek et al., 2009; Kovalchuk &amp; Driessen, 2010; Klein et al., 2011</td>
</tr>
<tr>
<td>Codes for a protein involved in sumoylation, reported to have an impact upon growth, morphology and stress response</td>
<td>Leach et al., 2011</td>
</tr>
</tbody>
</table>

*Information was edited from the CGD ([http://www.candidagenome.org/](http://www.candidagenome.org/))

### Table 2
Primers used in this study.

<table>
<thead>
<tr>
<th>PRIMER</th>
<th>SEQUENCE (5' TO 3') a, b, c</th>
<th>TARGET</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gene-specific primers</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ca_SNQ2-2F/ Ca_SNQ2-2R</td>
<td>AGAAACGGAGGATGTGGGAAGGT/ CTTCTTGAAAGAAGCTGTGATGAGG</td>
<td>SNQ2/ORF19.5759</td>
</tr>
<tr>
<td>Ca_HST6-2F/ Ca_HST6-2R</td>
<td>TGTTGTAGTGGTGGTGGTGCC/ CCAGAACATCAAATCCAGAAGGT</td>
<td>HST6/ORF19.7440</td>
</tr>
<tr>
<td>Ca_HYM1-796-1F/ Ca_HYM1-796-1R</td>
<td>TGTTGCACTGTGTGTTACGT/ GGTCACGGCCATTGGATGGT</td>
<td>HYM1/ORF19.796</td>
</tr>
<tr>
<td>Ca_4612-1F/ Ca_4612-1R</td>
<td>CTGTCAAGCTCTAATGTATGTTGACC/ AAGGACGGTGTGGTCACCGGG</td>
<td>ORF19.4612</td>
</tr>
<tr>
<td>Ca_5574-1F/ Ca_5574-1R</td>
<td>TTTTATTAGTTGGGAACGCCA</td>
<td>ORF19.5574</td>
</tr>
<tr>
<td>Ca_SMT3_670-1F/ Ca_SMT3_670-1R</td>
<td>ACCCAAACCATTGCCTCTTTTA/TG GAGGTATAGCATAATAGCACGCGT</td>
<td>SMT3/ORF19.670</td>
</tr>
<tr>
<td>Ca_SSY1_814-1F/ Ca_SSY1_814-1R</td>
<td>AGTGGGCTTGCTCAGAGTCA/ GTCTCCCATCCCCCTGT</td>
<td>SMT3/ORF19.670</td>
</tr>
<tr>
<td>Inverse primers</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ca_SNQ2-3F/ Ca_SNQ2-3R</td>
<td>TAGCATGCGGAGCACTCAATTGTGGT/ TAGGATGCTGGTTGTTGGTGGTCACGGCAGAGAGGT</td>
<td>5'3' flank</td>
</tr>
<tr>
<td>Ca_HST6-3F/ Ca_HST6-3R</td>
<td>TAGCATGCGGAGCACTCAATTGTGGT/ TAGGATGCTGGTTGTTGGTGGTCACGGCAGAGAGGT</td>
<td>5'3' flank</td>
</tr>
<tr>
<td>PRIMER</td>
<td>SEQUENCE (5' TO 3') a, b, c</td>
<td>TARGET</td>
</tr>
<tr>
<td>---------------------------</td>
<td>-----------------------------</td>
<td>------------</td>
</tr>
<tr>
<td>Ca_HYM1_796-2F/ Ca_HYM1_796-2R</td>
<td>TGGATGGTTGA</td>
<td>5'/3' flank</td>
</tr>
<tr>
<td>Ca_4612-2F/ Ca_4612-2R</td>
<td>TAGCATGCCTAGGTGGAGTTTGGG</td>
<td></td>
</tr>
<tr>
<td></td>
<td>GTTCT/TAGCATGCCCTAAAGGTC</td>
<td></td>
</tr>
<tr>
<td></td>
<td>TCAAAAGGTCACAG</td>
<td></td>
</tr>
<tr>
<td>Ca_5574-2F/ Ca_5574-2R</td>
<td>TAGCATGCACATTGGTGGGTTTGGGA</td>
<td></td>
</tr>
<tr>
<td></td>
<td>AGCCA/TAGCATGCAGTGGTTGCGCC</td>
<td></td>
</tr>
<tr>
<td></td>
<td>ATCACATTGGCA</td>
<td></td>
</tr>
<tr>
<td>Ca_SMT3_670-2F/ Ca_SMT3_670-2R</td>
<td>TAGCATGCTGGTGAGCTCCTGTG</td>
<td></td>
</tr>
<tr>
<td></td>
<td>GT/TAGCATGCTCACCCTGACACAAATTG</td>
<td></td>
</tr>
<tr>
<td></td>
<td>GCGGTTG</td>
<td></td>
</tr>
<tr>
<td>Ca_SSY1_814-2F/ Ca_SSY1_814-2R</td>
<td>TAGCATGCTGAGCCTGAAATCCTATC</td>
<td></td>
</tr>
<tr>
<td></td>
<td>TGACCCT/TAGCATGCTTTGGGATGG</td>
<td></td>
</tr>
<tr>
<td></td>
<td>AGGTCGAGCT</td>
<td></td>
</tr>
</tbody>
</table>

**SAT1 flipper primers**

| SAT1flipper-1F/ SAT1flipper-1R | GCATGCGATCTCTCTTAGGCTAGCCAT | SAT1 flipper |
|                                | GGAGGCGCAATTAACCTCACTAAA    |             |
|                                | GT/CATGCGATCTCTCTAGGCTAGCC |             |
|                                | CATGAGCGCGCGTAATACGACTCA    |             |
|                                | CTAT                        |             |

**Conformation of genomic integration**

| SAT1_Gene F1 | AACTAGACCTCAGTCTCGAAGCAGGAA | SAT1 gene C |

a The underlined bases represent the *Sphl* restriction site
b *SAT1* flipper primers contain multiple cloning sites (highlighted in grey)
c Inverse primers result in a linear vector with only flanking sequences

### 3.3 Genomic DNA isolation

Genomic DNA was isolated and purified from a 24 h *C. albicans* culture (50-100 mg wet weight), resuspended in 200 µL of distilled water, using the ZR Fungal/Bacterial DNA MiniPrep™ (Zymo Research) following the manufacturer’s recommended specifications. Alternatively, genomic DNA was isolated manually using the modified version of Than (2006). Briefly, *C. albicans* cells were cultivated on YPD broth overnight at 30 °C. Cells were harvested by centrifugation and resuspended in 1 mL of 2X saline-sodium citrate (SSC) [100 mL 20X (175.3 g/L NaCl, 88.2 g/L Na₂₃C₅H₇O₂⁻·2H₂O, pH 7) and 1 g sodium dodecyl sulphate (SDS) in 1 L ddH₂O] and heated at 99 °C for 10 min. Cells were collected and washed once with 1 mL sterile deionized water by centrifugation. To the cell pellet, 1 mL glass beads (0.2-0.5 mm in diameter), 1 mL chloroform and 200 µL of DNA-free (PCR) water were added. The tube was vigorously shaken for 10 min by vortexing and then centrifuged at 10 000 g for 10 min. The upper layer was transferred to a new microfuge tube and used directly as template for PCR reactions.
3.4 Polymerase chain reaction (PCR)

Open reading frames, along with ~500-bp flanking sequences, were amplified using polymerase chain reaction (PCR) based on different manufacturer’s protocols [e.g. Phusion® High-Fidelity PCR Master Mix with HF Buffer (New England BioLabs) or OneTaq® or OneTaq® Hot Start DNA Polymerase (New England BioLabs)] to generate blunt ends or T-overhangs on the terminal end of the PCR products, respectively. Expand Long Template PCR System (Roche Applied Science®) was used to amplify the SAT1 flipper which was provided in pBluescript. The flipper construct contains regions such as the nourseothricin (NTC) resistance gene, isolated from Streptomyces naourseri, which confers resistance to NTC, the flipase recombinase gene coding for the recombinase enzyme, the inducible maltose promoter (MAL2P) required for transcription of the recombinase gene as well as the flipase recombinase for recombination of the target sequences required for marker recycling (Reuss et al., 2004). Gel electrophoresis was performed for the PCR products and the SAT1 flipper, along with a molecular marker (O’GeneRuler DNA Ladder, Fermentas) and visualized on a 1% agarose gel (Agarose D1 LE, Whitehead Scientific, (Pty) Ltd). Samples were gel purified using BioSpin Gel Extraction Kit (Bioer Technology Co., Ltd) following manufacturer’s specifications and the purified PCR products were stored at -20 °C for future experiments. Sequencing of flanking regions was performed with the ABI BigDye Terminator Cycle sequencing kit (Applied Biosystems) and resulting sequences were analyzed using Geneious Pro 5 (Drummond et al. 2011).

3.5 Cloning of ORFs and the SAT1 flipper

DNA ligations were performed for cloning of blunt PCR products, which were firstly phosphorylated with a T4 polynucleotide kinase (Fermentas) (incubation for 20 min at 37 °C and kinase deactivation for 20 min at 80 °C using a PCR machine) into pSMART (Lucigen® Corporation). pGEM®-T easy (Promega® Corporation), which contains the ampicillin resistance gene, was used for cloning of PCR products containing T-overhangs as well as the SAT1 flipper. DNA ligations were catalyzed using 2 U/µL T4 DNA ligase with 10x buffer (Fermentas) and 3 U/µL T4 ligase with 2x buffer (Promega® Corporation) (Table 3) either overnight at 4 °C or for 3 h (pSMART) to 4 h (pGEM®-T easy) at 16 °C or at room temperature, followed by a PCR post-ligation step for 20 min at 80 °C to deactivate the ligase.

<table>
<thead>
<tr>
<th>Table 3</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Blunt-vector cloning (pSMART HC Kan)</strong> &amp; <strong>T-vector cloning (pGEM®-T easy)</strong></td>
</tr>
<tr>
<td><strong>Ligation buffer</strong></td>
</tr>
<tr>
<td><strong>T4 Ligase</strong></td>
</tr>
<tr>
<td><strong>Template DNA</strong></td>
</tr>
<tr>
<td><strong>Vector</strong></td>
</tr>
</tbody>
</table>
### Table

<table>
<thead>
<tr>
<th>Blunt-vector cloning (pSMART HC Kan)</th>
<th>T-vector cloning (pGEM®-T easy)</th>
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<tr>
<td>Nuclease free water</td>
<td>Up to 10 µL</td>
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<tr>
<td>Incubation</td>
<td>@RT or 16 °C for 3 h or 4 °C O/N</td>
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<tr>
<td>Inactivation of T4 ligase</td>
<td>80 °C for 20 min</td>
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*a* Blunt PCR products were phosphorylated prior to cloning into pSMART  
*b* 10x ligation buffer for 2 U/µL T4 ligase (Fermentas) and 2x ligation buffer (Promega) for 3 U/µL T4 ligase was used for blunt- and T-vector cloning respectively.

### 3.6 Transformation into *E. coli*

For DNA transformation we used the method described by Sambrook and colleagues (1989). Total ligation mix was added to *Escherichia coli* (XL 10) rubidium chloride (RbCl₂) competent cells. This was followed by incubation on ice for 1 h for *E. coli* cells to absorb the recombinant plasmid. The cells were incubated for 35 seconds at 42 °C, followed by another incubation step in ice-water slurry for 2 minutes. One (1) mL LB broth (5 g/L yeast extract, 5 g/L tryptone and 10 g/L NaCl) supplemented with 5 µL 5 M glucose plus 50 µL 1 M Mg²⁺ or 250 µL SOC media (20 g/L tryptone, 5 g/L yeast extract, 6 g/L NaCl and 5 g/L KCl) was added to the cells. The contents were incubated for 1 h at 37 °C and were spread with a sterile hockey stick on pre-warmed LB agar plates (5 g/L yeast extract, 5 g/L tryptone, 10 g/L NaCl and 16 g/L agar) supplemented with ampicillin (30 mg/mL), IPTG (Isopropyl β-D-1-thiogalactopyranoside, 24 mg/mL) and x-gal (bromochloroindoxyl galactosides, 20 mg/mL) (LB_Amp) for blue white screening of colonies transformed with pGEM®-Teasy or kanamycin (LB_Kan) plates for colonies transformed with pSMART HC kan vector, respectively. Plates were incubated overnight at 37 °C.

### 3.7 Vector isolation

The recombinant plasmid was extracted from 16 h old cells propagated either on LB broth (5 g/L yeast extract, 5 g/L tryptone and 10 g/L NaCl) supplemented with ampicillin (LB_Amp) or kanamycin (LB_Kan) after overnight growth (±16h) at 37 °C. Cells were harvested and resuspended in 200 µL of STET buffer (8% w/v sucrose, 5% Triton x-100, 50 mM EDTA, and 50 mM Tris-HCl pH 8.0) and the cell suspension was lysed with 5 µL (50 mg/mL) of lysozyme to break the cell wall. After lyses, the suspension was heated at 85 °C for 60 seconds, cooled on ice for 10 minutes and centrifuged for 15 minutes at V_max at room temperature in order to retrieve a pellet which was removed with a sterile toothpick. To the supernatant, sodium acetate (2.5 M, pH 5.2) and isopropanol was added and incubated for 10 minutes at -20 °C, centrifuged and washed with 70% ethanol. The recovered recombinant plasmid was dried and resuspended in 50 µL Tris-EDTA/RNase buffer [1 mL TE plus 5 µL RNase (10mg/mL)].
3.8 Vector modification

Inverse PCR was performed in order to introduce an *SphI* restriction site (GCATGCG) onto the non-coding regions, homologous to flanking regions of the ORFs, contained in the cloning vector, and to separate the ORFs from these flanking sequences. However, *in silico* analysis using Geneious Pro version 5 (Drummond et al., 2011), revealed that pGEM®-T easy contains an additional *SphI* site, apart from the one we wanted to introduce, suggesting that the corresponding flank will be cut-out during the next cloning steps. Therefore, a mutation was introduced at this site using two techniques, site-directed mutagenesis (SDM) and T4 DNA polymerase mutagenesis.

For SDM, we performed an inverse PCR using mutagenic primers pGEM®-Sphl delete F (AATTGGCCCGACGTCACATTCTCC) and pGEM®-Sphl delete R (GAGTCGTATTACAATTCACTGGCCGTCG). The inverse forward primer contained the mutation while the inverse reverse binds on the vector backbone ~25-bp downstream of the *SphI* site. During PCR, these primers will introduce a mutation in the target restriction site resulting in mutated PCR products. PCR is then followed by removal of the template (parental DNA) using *DpnI* which cuts DNA coming from methylation positive *E. coli* strains i.e. strains containing a DNA adenine methyltransferase (*Dam*) gene. The PCR product is then purified, self-ligated and transformed using the aforementioned protocols, followed by colony screening by digesting with *SphI*. Alternatively, the T4 polymerase (Fermentas) was used to mutate the *SphI* restriction site. During this method, the recombinant plasmid was digested with *SphI* and T4 polymerase added to the linearized plasmid DNA. The T4 polymerase fills-in the 5' overhang and removes the 3' overhang using exonuclease activity, leaving behind the blunt ends. The remaining overhang will then ligate with the other end of the vector, resulting in the mutated *SphI* site. Following ligation, the newly synthesized recombinant vector was transformed into *E. coli*, as previously described, and colonies were screened either by digesting with *SphI* after inverse PCR or by double digest.

3.9 Cloning of NTC<sup>R</sup> gene with flanking regions

Inverse PCR for all the recombinants was performed to introduce the *SphI* site onto the flanking regions and also for ligation of the *SAT1* flipper. PCR bands were analyzed on a 1% agarose gel and digested with *DpnI* to degrade the parental DNA. A band corresponding to the expected product size (~4.3kb) was cut-out and prepared for cloning. After cloning, the recombinant plasmid was purified with the BioSpin Plasmid DNA Extraction kit (BioFlux) and prepared for sequencing. Flanking sequences cloned into pGEM®-T easy and pSMART were sequenced using gene-specific primers. Primers were initially diluted to a final concentration of 3.2 pmol from a 100 pmol stock. Sequencing reactions were carried out using the ABI BigDye Terminator Cycling sequencing Kit (Applied Biosystems). Resulting sequences were edited and aligned, using Geneious Pro 5 (Drummond et al., 2011), against sequences representing flanking regions from the *Candida* Genome Data base. The recombinants were then linearized with *SphI* and the *SAT1* flipper, containing multiple restriction sites into pGEM®-T easy, was also digested with the same enzyme to produce compatible ends. These were then ligated and transformed as before. Clones were screened with whole cell PCR [Thermo Pol PCR systems (New England BioLabs)] using gene-specific primers and the plasmid was isolated and
digested with EcoRI and NotI for positive clones. The final output was SAT1 flipper cassette, flanked by the FRT sequences, cloned between the two flanking regions homologous to the flanking sequences of each of the target genes of the C. albicans diploid genome. A complete deletion cassette was obtained by PCR using gene-specific primers or by restriction digestion using PvuII (pGEM®-T Easy) and NruI (pSMART). These fragments were used directly to transform C. albicans competent cells.

3.10 Construction of Candida albicans deletion mutants

3.10.1 MIC of NTC for selection of Candida albicans

The Minimal Inhibitory Concentration (MIC) of NTC was determined on YPD plates containing varying concentrations of NTC. Candida albicans cells were initially cultivated on YM agar plates (10 g/L glucose, 3 g/L yeast extract, 3 g/L malt extract, 5 g/L peptone and 16 g/L agar) overnight and transferred to YDP plates for another overnight incubation. Resulting colonies were then inoculated on YPD broth for 5 h and spread on NTC plates containing 25 µg/mL, 30 µg/mL, 35 µg/mL, 40 µg/mL, 45 µg/mL and 50 µg/mL NTC as well as 100 µg/mL, 125 µg/mL and 150 µg/mL. Plates were incubated at 37 °C and inspected for three to five days for growth of NTC resistant colonies.

3.10.2 Preparation of yeast competent cells

Competent cells were prepared according to Ramon and Fonzi (2009) with a few modifications to the protocol. Briefly, C. albicans cells were cultivated on YPD medium and incubated for 24 h at 30 °C. Fresh cells were picked from the plate using a pipette tip and inoculated into 10 mL YPD broth in a 15 mL polypropylene tube and incubated on a shaker for 16-24 h. A 1:100 dilution was prepared into fresh 5 mL YPD broth contained in test tubes to achieve a cell density of approximately 2x10^6 cells/mL i.e. an OD600nm between 0.5 and 1.0 (cultivation for about 4-5 h). Cells were harvested by centrifugation for 5 min at 5000 x g at room temperature. The cell pellet was washed with 1 mL of PCR water or Phosphate Buffered Saline (PBS) and resuspended in 1 mL of 1X TE-LiAc prepared from 10X TE stock solution (100 mM Tris-HCl, 10 mM EDTA, adjusted to pH 7.5 and filter sterilized) and 10X LiAc stock solution (2 M lithium acetate adjusted to pH 7.5 with acetic acid and filter sterilized). The suspension was kept on ice until transformation or stored at 4 °C and used within two weeks.

3.10.3 Transformation of Candida albicans

Transforming DNA solution was prepared by mixing 5 µL of single-stranded carrier DNA (denatured at 99 °C for 10 min) with 5 µL of linear transforming DNA generated by PCR or restriction digest in a 1.5 mL microcentrifuge tube. This step was followed by addition of 50-100 µL of competent cells to the DNA solution and mixing by repeated pipetting. About 300 µL of PEG 40% (prepared by mixing 2.4 mL 50% PEG
with 300 µL of 10X TE and 300 µL of 10X LiAc), pre-warmed at 50 °C, was added to the transforming DNA solution and mixed by repeated pipetting. This suspension was incubated stationary for 30 min at 30 °C and placed in 42 °C water bath for 30 min to heat shock the cells. Cells were then collected by centrifugation at room temperature for 4 min at 4000 x g and the pellet resuspended in YPD broth and incubated for 24 h to express the SAT1 gene. Cells were then washed once with and resuspended in 200 µL 1 M sorbitol. Approximately 100-150 µL of this suspension was spread on 150 µm/mL NTC containing plates and incubated for 2-3 days at 30 °C (Reuss et al., 2004; Ramon & Fonzi, 2009). Resultant colonies were streaked on YPD plates supplemented with 200 µm/mL NTC and further incubated for 48 h.

4. Results and discussions

4.1 PCR and cloning
Sequences of the ORFs, representing C. albicans genes used in this study, were retrieved from the Candida genome database (www.candidagenome.org/). DNA was purified from C. albicans (NRRL Y-27077) and seven genes (SNQ2, HST6, SSY1, HYM1, SMT3, ORF19.5574 and ORF19.4612) were amplified, along with their upstream and downstream flanking regions (Fig. 1). PCR products were gel purified and prepared for cloning.

The vector that was initially selected for cloning was the pSMART vector based on the manufacture’s recommendations that it minimizes cloning bias and maximizes competence, since the cloning process of pSMART vector is blunt with the ends being dephosphorylated hence it is unlikely to self-ligate or ligate with fragments lacking a phosphate group at the 5’ and 3’ ends. However, empty plasmid backbones (Fig. 2) were obtained. As a result, we succeeded to clone only the small genes (HYM1, ORF19.4612 and SMT3) into this vector (Fig. 3), but failed to clone the larger genes. As an alternative, we re-amplified the remaining genes (SNQ2, HST6 and ORF19.4612) in order to introduce cohesive ends at their 3’ and 5’ ends of the PCR products and cloned them, together with the SAT1 flipper, into pGEM®-T easy vector (Figs. 3 and 4).
Fig. 2. Illustrative example showing an empty (insert-free) pSMART vector after ligation of five different inserts.

Fig. 3. 1% agarose gel of *C. albicans* open reading frames cloned into a vector. SNQ2 (~5-kb), HST6 (~4.5-kb), SSY1 (~3.2-kb), ORF19.5574 (~2-kb) were cloned into pGEM®-T easy and confirmed by cutting with *Not*I while HYM1 (~1.9-kb), ORF19.4612 (~1.7-kb) and SMT3 (~670-bp) were cloned into pSMART and confirmed by cutting with *EcoR*I.

Fig. 4. 1% agarose gel of the SAT1 flipper (~4.3-kb) cloned into pGEM®-T easy and confirmed by cutting with *Not*I (Lane two).
4.2 Site directed mutagenesis

In this study, pSMART HC Kan was selected as the cloning vector since it is small, blunt and eliminates cloning bias. As a result, we selected an Sphl site as the restriction recognition sequence that will be sued to replace ORFs with the SAT1 flipper (containing the selectable marker, SAT1). As explained in the previous section, the only genes cloned into this vector were HYM1, ORF19.4612 and SMT3 and the remaining ORFs were cloned into the T-overhang vector, pGEM®-T Easy. To clone the SAT1 flipper into pGEM®-T Easy, we mutated the undesirable Sphl site, located in the multiple cloning site in all the recombinant plasmids. In the presence of this restriction site, the nearby flank is cut-out from pGEM®-T easy (Fig. 5) when creating compatible ends between the cloning vector and the SAT1 flipper and therefore, the deletion construct cannot be completed. Note that the presence of this additional Sphl site was only realised after the amplified C. albicans genes containing the ~500-bp flanking regions was cloned into pGEM®-T easy. Using site-directed mutagenesis with T4 DNA polymerase, we succeeded in creating a mutant vector which still contains the cloned C. albicans genes but minus the undesirable Sphl site. The modified recombinant plasmids were inversely amplified and the deletion of the Sphl site was confirmed by digesting with the same restriction enzyme (Fig. 6). If the Sphl site was successfully removed, no ~500-bp should be present as can be seen in Fig. 6. This allowed cloning of the SAT1 flipper using the Sphl restriction site that was inserted following inverse PCR.

![Fig. 5. Gel photo showing the flanking regions, contained in pGEM®-Teasy vector after linearizing using inverse PCR and digesting with Sphl. One flank is located between 500-600-bp while the other is still attached to the cloning vector.](image-url)
Fig. 6. Gel photo showing the flanking regions, contained in the cloning vectors after digesting with SpfI and treatment with DpnI. A band representing a cut out flank is no longer visible, indicating that the SpfI site is mutated on pGEM®-T easy backbone.

4.3 Designing the SAT1 knockout plasmids
For construction of the final deletion cassettes inverse primers (Table 2) were used where, following amplification, the ORF of each of the cloned genes where replaced with a SpfI restriction site. PCR products were self-ligated and confirmation of the removal of the ORFs was obtained through digestion using NotI for pGEM®-T Easy and EcoRI for pSMART. These two enzymes were selected seeing that they will remove any fragment cloned into these plasmids. In this case the resulting gel profile should indicate two bands representing the plasmid backbone and the flanking regions coupled together via an SpfI site (Fig. 7). It can be noted from Fig. 7 that the flanking sequences display varying sizes; for instance, the flanking regions of SNQ2, SSY1, ORF19.4612 and SMT3 are approximately 1-kb in size while the flanking regions for HST6 and ORF19.5574 are approximately 1.2-kb.

Fig. 7. Flanking regions separated from the open reading frames by inverse PCR. The cloning vector was self-ligated and digested with NotI (pGEM®-T easy) and EcoRI (pSMART; Lane 5 - 7).
In addition to restriction analysis all recombinant plasmids were also sequenced to confirm the presence of the correct flanking regions and to also confirm the presence of the SpfI cutting site between these sequences. In the sequencing results (Fig. 8), there are two scenarios; one is that only one SpfI site is present (HST6, SSY1, ORF19.5574 and ORF19.4612) and this can be, hypothetically, attributed to overlapping of sequence ends. In another scenario, two SpfI sites are observed (SNQ2, HYM1 and SMT3) which rules out overlapping of sequences and cloning of blunt ends. For the purpose of cloning the SAT1 flipper construct there will be no difference whether one or two SpfI site are present.

Fig. 8. Diagram of sequence assemblies showing part of the down- and upstream flanking sequences of SNQ2, HST6, SSY1, ORF19.5574, HYM1, ORF19.4612 and SMT3, separated by SpfI cutting site(s) (arrows) introduced by inverse PCR. Assemblies were generated using Geneious version 6.0 created by Biomatters (www.geneious.com).

Fragments which contain compatible cohesive ends generated by cutting with the same enzyme (non-directional cloning) often result in self-ligation after cloning. As a result, a lot of negatives are selected and positives are difficult to detect. Dephosphorylation was used to prevent self-ligation and thereby reducing the number of false positives during PCR screening. Cloning of the SAT1 flipper, to complete the deletion cassette, was confirmed in pGEM®-T Easy and pSMART using EcoRI (Fig. 9). The complete deletion cassette was linearized by amplification from the vectors using locus specific primers (P1/P2) or by cutting with PvuII (pGEM®-T Easy clones) and NruI (pSMART clones) (gel photo not shown); the linear construct was in turn used directly to transform C. albicans wild type strain. In addition, we determined the orientation of the SAT1 flipper in the vector after cloning by amplifying the SAT1 gene using one gene-specific (e.g. P1 or P2) and Sat1 1F primer designated P3. We could obtain a PCR product (~5-kb) using this primer combination (Fig. 9C).
**Fig. 9.** A diagram showing a simplified *in silico* map (not drawn to scale) of the SAT1 flipper cassette, flanked by *C. albicans* non-coding sequences (flanks), and the position of gene-specific (P1 and P2) and the SAT1 flipper primers (P3 = Sat1 1F and P4 = SAT1 1R) (A). Deletion constructs were cut from the cloning vectors using EcoRI (B) and their orientations were determined using two primer combinations, P1 vs. P3 (C).

### 4.4 Construction of *Candida albicans* deletion mutants

Reuss and co-workers (2004) transformed *C. albicans* wild type strain using the SAT1 flipper strategy in which alleles of the *OligoPeptide Transport1* (*OPT1*) were inactivated. In their study, they included a pre-selection step where the SAT1 gene was expressed in YPD broth for 4 h, followed by selection on 200 µg/mL NTC YPD plates. Nourseothricin resistant isolates were recovered within 24 h of incubation. Roemer et al., (2003) expressed the SAT1 gene overnight and selected on 400 µg/mL NTC YPD plates and recovered resistant isolates within 48 h of incubation. In the current study, the first attempt of transformation did not include a pre-selection step and both NTC resistant and sensitive isolates were obtained within 3 days at 30 °C after transforming with the SAT1 cassette. In the second attempt, a pre-selection step carried out for 24 h produced NTC resistant colonies within the second day of incubation. Isolates were picked and inoculated into YPD broth (supplemented with 200 µg/mL NTC) on roller drums overnight for genomic DNA isolation. Successful integration into *C. albicans* genome was confirmed for HST6, SSY1, ORF19.5574 and ORF19.4612 whereby a combination of gene-specific and the SAT1 cassette internal primer i.e. P2 vs. P5, was used to give a ~0.8 kb band (Fig. 10), indicating that the first copy of these genes was deleted. After isolates of ∆hst6, ∆ssy1, ∆orf19.5574 and ∆orf19.4612 were selected and grown overnight in 2% maltose broth at 30 °C, excision of the SAT1 cassette was confirmed by PCR using primers P1 and P2 which resulted in the amplification of the complete ORF and flanking regions indicated by ~1-Kb band (Fig. 10A).
All heterozygous mutants were cultured on YPD agar for 3-7 days to analyze and exclude phenotypic inconsistencies. From those isolates that displayed stable phenotypes, a second round of transformation was conducted on one of the isolate to delete second copies of the target gene. However, deletion of second copies of the target could only be confirmed for HST6 and SSY1 (Fig. 10) and not ORF19.5574 and ORF19.4612. One possible reason for our inability to obtain homozygous mutant for these genes is the chance that they were essential for, and their deletions could be lethal to the cells, on the chosen selection conditions. Sequences representing SNQ2, HYM1 and SMT3 were also not confirmed for deletion due to technicalities in transformation. For both single and double deletions that we performed, two or more isolates of Δhst6 and ΔΔhst6, Δssy1 and ΔΔssy1, Δorf19.5574 as well as Δorf19.4612 for C. albicans NRRL Y-27077 were kept at -80 °C in 15% glycerol until the next experiment. These isolates were also cryopreserved at -196 °C in the UNESCO-MIRCEN Biotechnological Yeast Culture Collection, Department of Microbial, Biochemical and Food Biotechnology (Table 4).
**Fig. 10.** PCR analysis showing the insertion of the SAT1 flipper cassette after first (A) and second round (B) of transformation into C. albicans genome. The *in silico* map displayed on top of the gel photos is illustrative of expected PCR product sizes.
Table 4.  
*Candida albicans* mutant strains constructed from *C. albicans* NRRL Y-27077.

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<th>UOFS Y- Strain</th>
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Strains are stored in the UNESCO MIRCE M Culture Collection
5. Conclusions and future research

This part of the study entailed construction of a number of deletion constructs with the purpose of obtaining homozygous deletion mutants of *C. albicans*. A dominant selectable marker, the SAT1 gene, was cloned between an approximately 500-bp up and downstream region of the target genes and this construct was used to delete *HST6*, *SSY1*, *ORF19.5574* and *ORF19.4612* to generate loss of function in the mutant strains. The utility of the SAT1 flipper overcomes roadblocks encountered using conventional nutritional markers such as the need to use an auxotrophic strain (Fonzi & Irwin, 1993; Reuss et al., 2004).

Using the SAT1 flipper, deleting the first copy of four of the target genes, *HST6*, *SSY1*, *ORF19.5574* and *ORF19.4612*, was a success without including any pre-incubation steps to express the SAT1 DNA (Roemer et al., 2003; Reuss et al., 2004). Nonetheless, our attempt to delete the second copy of *ORF19.5574* and *ORF19.4612* did not yield homozygous deletion mutants even when we included a pre-incubation step of either four hours (Reuss et al., 2004) or overnight (Roemer et al., 2003). We reason that these genes may be essential under the selection conditions used in this study. More attempts are underway to knock out the second copy of these genes and this time a different approach will be attempted. For instance, the second copy of *ORF19.5574* and *ORF19.4612* can be placed under the control of a regulatable promoter such as Met3p (Care et al., 1999) where in which, their expression can be turned on and off to analyze resulting phenotypes.

Integration of the deletion cassette in the place of the target genes was confirmed by amplifying a part of the cassette (SAT1 DNA) and one of the flanking sequences of the target gene. This method is desirable in that it allows screening of many isolates. However, determining PCR conditions of primers from different sets can be tedious and time consuming. In addition, primers may anneal in a way that undesired bands are produced alongside the desired one and this may complicate interpretation of the results. To overcome this problem, we screened more isolates to ensure that this was indeed non-specific binding and not a problem with the designed deletion construct.

Literature advocates that for reliable phenotypic analysis, additional isolates can be used to study the effects of deleting the target gene and to exclude undesired genomic integration of the deletion cassette (Noble & Johnson, 2007; Noble et al., 2010). Using more than one isolate which represents each constructed mutant strain also assists in alleviating phenotypic inconsistencies (Homan et al., 2009). Literature also strongly advocates that a complement strain with the restored gene function be included during phenotypic analysis (Noble & Johnson, 2007). However, complemented genes also present a problem of unpredictable expression levels and analogous unpredictable phenotypic outputs (Noble & Johnson, 2007). Perhaps reliable phenotypic analysis can result from applying both approaches i.e. use of additional isolates of a mutant and that of complemented mutant strains which may require time and could prove cost effective when complex assays are included. So far, we have designed single and double deletion mutant strains of *HST6*, *SSY1*, *ORF19.5574* and *ORF19.4612* and preserved at least more than one isolate for each strain. Construction of complement mutant strains is currently underway in our laboratory and these strains will be used to further validate important phenotypes displayed by the deletion mutants.
6. References


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Identification by gas chromatography–tandem mass spectrometry and quantification by enzyme immunoassay. Molecular Immunology, 49 621-627.


Chapter III – The Basic Biological Functions of Candida albicans Hst6p: Evidence for a Role in Stress Response and Yeast-Hyphal Transition

(Submitted for review in the journal, Fungal Genetics and Biology; work presented follows the format of this journal)

Abstract

Candida albicans is a commensal yeast of humans that can display a set of phenotypes allowing it to infect different niches within the human host. In order to do this successfully, this pathogen has evolved mechanisms of coping with different stresses encountered in the host micro-environment. In this study we demonstrate that the membrane bound protein, Hst6p, member of the ATP binding cassette family of transport proteins, is involved in stress response by characterizing a homozygous deletion mutant, C. albicans Δhst6. Previously, this gene was found to be significantly up-regulated during growth in the presence of the polyunsaturated fatty acid, arachidonic acid. According to high throughput phenotypic microarrays (PMs), Δhst6 displayed great sensitivity to 93% of stress inducing compounds, while the wild type strain was less or moderately sensitive. Consistent with this data, conventional phenotypic assays confirmed that this mutant was sensitive to oxidative, osmotic, cell wall, heavy metal and heat stress. In addition, in the presence of excess nitrogen source, filamentous growth was observed for Δhst6, but not the wild type strain. This suggested that Hst6p suppress filamentation in the presence of excess nitrogen. Therefore, we propose that Hst6p plays a role in C. albicans stress response as well as nitrogen source dependent morphogenesis.

Key words: ABC transporter · Amino acid · Filamentation · Morphogenesis · Phenotypic microarray · Stress response
1. Introduction

*Candida albicans* is a common member of the human microbiome. Although it is a human commensal it can cause superficial infections and life-threatening diseases. Pathogenesis in *C. albicans* strains is generally reflective of impaired host immunity where they respond by activating virulence factors, causing systemic infections and life threatening diseases such as Candidemia and Candidiasis. The interconnected transcriptional circuit that this yeast utilizes within the host for colonization has recently been described (Pérez et al., 2013; Pérez & Johnson, 2013). The ability of *C. albicans* to colonize and cause infection implies that the yeast can adapt to varying stresses exerted by the host micro-environment. These include osmotic and thermal shock, oxidative and nitrosative stress, heavy metal stress and cell wall damage as well as treatment with antifungals such as azole drugs (Brown et al., 2014; Harrison et al., 2014). Some of the mechanisms for dealing with these stresses include the activation of the mitogen-activated protein (MAP) kinase pathway to cope under osmotic, oxidative, cell wall and heavy metal stress and the Cta4-Yhb1 as well as the Hsp90-Hsf1 signaling pathway to cope under nitrosative stress and thermal pressures respectively (Brown et al., 2014). Moreover, *C. albicans* also developed mechanisms to cope under nitrogen starvation by increasing expression of membrane-bound sensors such as ammonium permeases (e.g. Mep1p and Mep2p) (Biswas & Morschhäuser, 2005; Dunkel et al., 2014). These proteins drive hyphal formation in this yeast to obtain nutrients from distant localities and, in addition, promote pathogenesis by permitting protease secretion (Dunkel et al., 2014).

Current knowledge indicates that exposure of *C. albicans* to polyunsaturated fatty acids can cause an increase in membrane fluidity, changes in ergosterol content as well as oxidative stress, leading to apoptosis and increased susceptibility to antifungal drugs (Eells et al., 2009; Thibane et al., 2012; Mishra et al., 2014). Previous work in our research group reported that the gene for the membrane-bound Hst6p was significantly up-regulated during growth of *C. albicans* biofilms in the presence of exogenously supplemented arachidonic acid (AA) (Eells, 2011). The *HST6* gene is a homologue of *Saccharomyces cerevisiae* STE6 and its encoded protein is the ABC transporter responsible for the secretion of the mating factor a pheromone (Mfa) (Raymond et al., 1998; Bauer et al., 1999; Magee et al., 2002). In *C. albicans*, Hst6p is expressed in the yeast cells of the white and opaque phenotype, as well as in hyphal cells (Raymond et al., 1998) and biofilms (Eells, 2011). The fact that *C. albicans* *HST6* gene expression is not phenotype-specific implies that its protein may possess a more generic function. To further explore this possibility, the role of Hst6p in *C. albicans* stress response as well as nitrogen source dependant yeast-hyphal switching is revealed in this chapter.

2. Materials and methods

2.1 Strains used

*Candida albicans* NRRL Y-27077, isolated from a skin lesion was obtained from the Agricultural Research Services Yeast Culture Collection (http://nrnl.ncaur.usda.gov) and frozen as glycerol stocks in liquid nitrogen until use. After revival, working
cultures were obtained on yeast-extract-peptone-dextrose (YPD) agar (10 g/L yeast extract, 20 g/L peptone and 20 g/L glucose and 20 g/L agar). Deletion of HST6 was conducted as previously described (Chapter II: section 3 and 4) and the independent ΔΔhst6 isolates, UOFS Y-2879 and UOFS Y-2880, were used in this study.

2.2 High throughput phenotypic microarrays

To conduct phenotypic microarrays (PMs), the wild type strain and one ΔΔhst6 isolate (UOFS Y-2879) were cultivated at 30 °C for 24 h on YPD agar. A single colony from this agar was streaked on Biolog Universal Growth Agar (BioLog, USA), supplemented with 5% (w/v) sheep blood, and incubated for another 24 h at 30 °C after which, cells were removed by a sterile swap and inoculated in sterile water. Cells were adjusted to 62% transmittance (~5x10⁶ cells/mL) using a Turbidimeter (BioLog, USA) and added accordingly to inoculating fluids (Table 1). Inoculation fluids, along with cells from the wild type and mutant strain, and the Biolog Redox (tetrazolium) Dyes, included to measure cellular respiration, as well as yeast nutrient supplement (Table 1) were inoculated (100 µL) in the 96-well PM assay plates (PM01-10) pre-warmed at room temperature. Following this, plates were placed in the OmniLog incubator (BioLog, USA) (at 30 °C) for a 48 h incubation period. Each PM microplate comprised 96 assays for carbon utilization (PM01-02), nitrogen utilization (PM03, PM06-08), phosphorus-sulphur utilization (PM04), biosynthetic pathway/nutrient stimulation (PM05), osmotic/ionic response (PM09) and pH response (PM10). The OmniLog reader takes readings by capturing the formazan colour intensity in each well every 15 minutes in order to provide a signal value (formed from pixel density) reflecting cell respiration and dye reduction. Formazan is formed during reduction of the compound, tetrazolium, resulting in a purple colour. After incubation, the raw kinetic data were imported into the Data Management File Management/Kinetic Analysis, and Parametric Analysis software packages (v1.6) (BioLog, USA) for further analysis. Standard curves generated by the OmniLog reader were reflective of the tetrazolium dye reduction which was in turn reflective of cellular respiration. Values from the standard curves were then exported into Microsoft Excel for further analysis.

Table 1

Procedure for inoculation into phenotypic microarrays.

<table>
<thead>
<tr>
<th>PM stock solution</th>
<th>PM01,02 (mL)</th>
<th>PM03,06,07,08 (mL)</th>
<th>PM04 (mL)</th>
<th>PM05,09 (mL)</th>
<th>PM10 (mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>IFY-0 (1.2x)</td>
<td>20.0</td>
<td>40.0</td>
<td>10.0</td>
<td>20.0</td>
<td>-</td>
</tr>
<tr>
<td>SC Medium (1.2x)</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>100.0</td>
</tr>
<tr>
<td>Dye mix D (75x)</td>
<td>0.32</td>
<td>0.64</td>
<td>0.16</td>
<td>0.32</td>
<td>-</td>
</tr>
<tr>
<td>Dye mix E (100x)</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>1.20</td>
</tr>
<tr>
<td>D-glucose (32x)</td>
<td>-</td>
<td>1.50</td>
<td>0.375</td>
<td>0.75</td>
<td>3.75</td>
</tr>
<tr>
<td>PM additive (12x)</td>
<td>2.0</td>
<td>4.0</td>
<td>1.0</td>
<td>2.0</td>
<td>0</td>
</tr>
<tr>
<td>Cells</td>
<td>0.5</td>
<td>1.0</td>
<td>0.25</td>
<td>0.5</td>
<td>2.5</td>
</tr>
<tr>
<td>Sterile water</td>
<td>1.18</td>
<td>0.86</td>
<td>0.215</td>
<td>0.43</td>
<td>12.55</td>
</tr>
<tr>
<td>Total</td>
<td>24.0</td>
<td>48.0</td>
<td>12.0</td>
<td>24.0</td>
<td>120.0</td>
</tr>
</tbody>
</table>
Final concentration of ingredients in PM inoculating fluids after the addition of cell suspension

<table>
<thead>
<tr>
<th>Ingredient</th>
<th>PM01,02 (mM)</th>
<th>PM03,06,07,08 (mM)</th>
<th>PM04 (mM)</th>
<th>PM05,09 (mM)</th>
<th>PM10 (mM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>IFY</td>
<td>1x</td>
<td>-</td>
<td>-</td>
<td>1x</td>
<td>-</td>
</tr>
<tr>
<td>SC Medium</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>1x</td>
</tr>
<tr>
<td>Dye mix D</td>
<td>1x</td>
<td>1x</td>
<td>1x</td>
<td>1x</td>
<td>-</td>
</tr>
<tr>
<td>Dye mix E</td>
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<td>-</td>
<td>-</td>
<td>-</td>
<td>1x</td>
</tr>
<tr>
<td>D-glucose</td>
<td>-</td>
<td>100</td>
<td>100</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>L-glutamic acid</td>
<td>5</td>
<td>5</td>
<td>-</td>
<td>5</td>
<td>-</td>
</tr>
<tr>
<td>Potassium phosphate</td>
<td>5</td>
<td>5</td>
<td>-</td>
<td>5</td>
<td>-</td>
</tr>
<tr>
<td>(pH 6.0)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Sodium sulfate</td>
<td>2</td>
<td>2</td>
<td>-</td>
<td>2</td>
<td>-</td>
</tr>
<tr>
<td>Adenine HCl monohydrate</td>
<td>0.05</td>
<td>0.05</td>
<td>0.05</td>
<td>0.05</td>
<td>0.05</td>
</tr>
<tr>
<td>L-histidine HCl</td>
<td>0.01</td>
<td>0.01</td>
<td>0.01</td>
<td>0.01</td>
<td>0.01</td>
</tr>
<tr>
<td>monohydrate</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>L-leucine</td>
<td>0.1</td>
<td>0.1</td>
<td>0.1</td>
<td>0.1</td>
<td>0.1</td>
</tr>
<tr>
<td>L-lysine</td>
<td>0.05</td>
<td>0.05</td>
<td>0.05</td>
<td>0.05</td>
<td>0.05</td>
</tr>
<tr>
<td>L-methionine</td>
<td>0.025</td>
<td>0.025</td>
<td>0.025</td>
<td>0.025</td>
<td>0.025</td>
</tr>
<tr>
<td>L-tryptophan</td>
<td>0.025</td>
<td>0.025</td>
<td>0.025</td>
<td>0.025</td>
<td>0.025</td>
</tr>
<tr>
<td>Uracil</td>
<td>0.03</td>
<td>0.03</td>
<td>0.03</td>
<td>0.03</td>
<td>0.03</td>
</tr>
</tbody>
</table>

SC medium (1.2x) is composed of filter sterile 8.04g/L yeast nitrogen base and 2.4g/L synthetic complete mixture.

These are the PM additive solutions (prepared from 12x stock solutions) indicated in light green. Yeast nutrient supplement (indicated in tan) was prepared from 48x stock solutions.

2.3 Stress induction

The wild type strain and two independent isolates of ΔΔhst6 were initially plated onto YPD agar and incubated for 24 h at 30 °C. Cells were then resuspended in 5 mL phosphate buffered saline (PBS) (Sigma-Aldrich, USA) and diluted with the same buffer to an OD_{600 nm} of 0.08. Cells were serially diluted (1:100), spotted on YPD agar supplemented with various stresses listed in Table 1. For stress tests using YPD agar, duplicate plates were incubated at 30 °C (unless indicated otherwise in Table 1) for three to seven days after which photos were taken and processed for analysis of colonies displaying stress related phenotypes. Nitrosative stress was determined by streaking cells (in PBS) onto duplicate plates containing 40 mM S-Nitrosoglutathione (GSNO) followed by incubation at 30 °C for 3-7 days.

Table 1

Stress-inducing conditions used in this study.

<table>
<thead>
<tr>
<th>Stress</th>
<th>Inducing condition(s)</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>Temperature</td>
<td>Incubation at 30 °C, 37 °C and 42 °C</td>
<td>Homann et al., 2009, Leach et al., 2011, 2012</td>
</tr>
<tr>
<td>Heavy metal</td>
<td>Lithium (300 mM LiCl), Zinc (10 mM ZnSO₄)</td>
<td>Homann et al., 2009</td>
</tr>
<tr>
<td></td>
<td>Copper (15 mM CuSO₄); Iron (15 mM FeCl₃)</td>
<td></td>
</tr>
<tr>
<td>Cell wall</td>
<td>Caffeine (15 mM)</td>
<td>Homann et al., 2009</td>
</tr>
<tr>
<td></td>
<td>Sodium dodecyl sulphate (SDS, 0.08%)</td>
<td></td>
</tr>
<tr>
<td>Oxidative</td>
<td>H₂O₂ (6 mM)</td>
<td>Homann et al., 2009, Leach et al., 2011, 2012</td>
</tr>
</tbody>
</table>
2.4 Colony and cellular morphology on solid media containing different nitrogen sources

The wild type and \( \Delta \Delta hst6 \) (UOFS Y-2879 and UOFS Y-2880) strains were inoculated from a glycerol stock on YPD agar plates for 10 days at room temperature. A loopful of cells was inoculated in YPD broth and incubated for 24 h at 30 °C on roller drums. Cells were harvested by centrifugation and washed twice with, and resuspended in 5 mL PBS. This culture was then inoculated into minimal media (M9), supplemented with 1% glucose, without a nitrogen source for 48 h for nitrogen starvation. Optical density (OD) readings were taken and standardized to OD\(_{600}\) nm of 0.08 with PBS. From a 1x and 5x dilution of this suspension, 3 µL was spotted onto M9 solid medium (64 g/L \( \text{Na}_2\text{HPO}_4 \cdot 7\text{H}_2\text{O} \), 15 g/L \( \text{KH}_2\text{PO}_4 \), 2.5 g/L NaCl, 5 g/L (NH\(_4\))\(_2\)SO\(_4\) and 14 g/L agar) and on M9 without (NH\(_4\))\(_2\)SO\(_4\) supplemented with only 1% glucose to serve as a control or with 100 µM or 10 mM filter sterilized amino acids (L-ornithine, L-lysine, L-alanine, L-proline, L-isoleucine, L-threonine, L-methionine and L-leucine). All plates were incubated at 25 °C, 30 °C and 37 °C for up to 14 days. Photos were taken with the gel documentation system (Bio-Rad XRS, Quantity One 4.5.0) after 3, 7 and 14 days to analyze colony morphology on agar. Cellular morphology of non-invasive cells was determined at 1000X magnification using a Zeiss Axioplan light microscope. Agar invasiveness was determined by washing off non-invasive cells with a gentle stream of water. The ability of hyphae to penetrate the agar surface was visualized by generating grey scale images using the gel documentation system.

2.5 Germ tube assays

The ability of the wild type and mutant strains to form hyphae was determined by examining formation of germ tubes in liquid cultures. Cells from a glycerol stock were cultured in YPD broth for 16 h at 30 °C. Following this, cultures were harvested, washed with, and resuspended in 5 mL PBS. This suspension was diluted to an OD\(_{600}\) nm of 0.1 into liquid media containing amino acids, i.e. YPD supplemented with 10% Fetal Bovine Serum (Sigma-Aldrich, USA), Spider medium (Liu et al., 1994) and Lee’s medium (Lee et al., 1975) modified according to Lu and co-workers (2011) supplemented with 1.25% glucose (Glc) or N-acetylglucosamine (GlcNAc), and in media without amino acids, i.e. M9 supplemented with 1.25% Glc or GlcNAc. Cells were incubated for 18 h at 37 °C and hyphal production was determined. Cells (1000-1500) from ten random fields generated by a camera from three separate experiments were counted at 400x magnification using a Zeiss Axioplan light microscope. In addition, a germ tube assay was conducted according to Ells and co-workers (2011) with a few modifications. Briefly, a loop full of cells, grown on YPD agar plates for 24 h at 30 °C, was diluted to an OD\(_{600}\) nm of 0.1 in filter sterilized RPMI-1640 medium (which contain low concentrations of amino acids) with or without 120 pg/mL prostaglandin E\(_2\) (PGE\(_2\)) (Cayman Chemicals, USA). After
incubation at 37 °C for 4 h, 1000 to 1500 cells were visually assessed for germ tube formation as previously mentioned.

2.6 Statistical analysis

All experiments, except phenotypic microarrays, were performed in triplicate unless stated otherwise. A student t-test was conducted to determine the statistical significant difference between data sets.

3. Results and discussions

3.1 Response to environmental stress

3.1.1 Analysis based on high throughput phenotypic screening

High-throughput analyses of biological samples using phenotypic microarrays (PMs) has become increasingly important in dissecting the metabolic impacts of mutants in yeasts, fungi, bacteria and animal cell lines (Bochner et al., 2001). PMs can profile up to 1000 phenotypes colorimetrically within 48 h at physiological temperatures based on respiration capacity of isolates measured by tetrazolium dye reduction. The intensity of the purple colour as result of formazan formation is generated by biochemically respiring cells and converted into values that are plotted into kinetic curves by the Omnilog reader. Since PMs rely on cell respiration measurements, growth-independent respiration can be detected in cases where such phenotypes occur (Bochner et al., 2001).

We used PM assays as an initial screen for phenotypes resulting from the deletion of HST6. Our results indicated that the most striking differences between the wild type and ΔΔhst6 were in the reaction to ~93% (90/96) of stress-inducing compounds in the PM09 microplate (Fig. 1). Phenotypic data generated using PM09 showed that ΔΔhst6 was extremely susceptible to the stress conditions in this plate, compared to the wild type strain which displayed high respiration levels (Fig. 1A-F), suggesting that Hst6p plays an important role in providing protection against osmotic/cationic stress. Since HST6 encodes an ABC transport protein (Decottignies & Goffeau, 1997; Raymond et al., 1998), we reasoned that this protein may be responsible for exporting compounds which may accumulate inside cells to stressful levels. To closely analyze the observed PM responses, we divided the data in the PM09 plate into different types of responses and/or stresses i.e. osmotic (salt) stress, osmoprotectants and response to organic and inorganic salts. Although a general dose-dependent decrease in response to salt concentration was observed for the wild type strain, ΔΔhst6 was sensitive to all concentrations of NaCl after 24 h (results not shown) and 48 h (Fig. 1A). The addition of osmoprotectants did not rescue ΔΔhst6 from this sensitivity (Fig 1B). Interestingly, although the wild type strain was slightly sensitive to the preservative organic salt, sodium benzoate, at concentrations higher than 50 mM, ΔΔhst6 was highly sensitive to this compound at concentrations above 20 mM (Fig. 1C). In the case of the inorganic salts, Na2SO4, Na2PO4, (NH4)2SO4, NaNO3 and NaN02, ΔΔhst6 was more sensitive than the wild type strain.
after 48 h (Fig. 1D-F). This initial phenotypic screen suggests that Hst6p may protect 
*C. albicans* from a range of stress-inducing organic and inorganic compounds with
antimicrobial properties.

Fig. 1. Analysis of high throughput phenotypic data using PM09 of the OmniLog
system after 48 h at 30 °C. A. Osmotic stress analysis based on sodium chloride. B.
Effects of osmoprotectants against 6% NaCl. C-F. Sensitivity against organic and
inorganic preservative salts.

3.1.2 Conventional phenotypic screening

Since PM assays were not repeated, we validated results obtained from this initial
phenotypic screen by conducting a series of stress response experiments using
YPD agar. To validate whether the observed phenotypes are indeed the result of the
deleted gene and not the result of non-specific integration of the deletion cassette that may occur during genetic transformations, a complemented strain i.e. a strain with the restored allele(s), or two independent isolates of the mutant should be used (Homann et al., 2005, 2009; Noble et al., 2010). In addition, additional isolates may be used to resolve phenotypic inconsistencies between isolates of the mutant strain (Homann et al., 2009). In this study, we utilized two independent isolates of ΔHst6 in all the conventional stress assays to validate observed phenotypes. However, since similar results were obtained for the mutant strains, only the results of one isolate are shown (Fig. 2). These results confirmed that ΔHst6 was sensitive to osmotic pressure due to NaCl and, in addition, to sucrose. Further, ΔHst6 displayed sensitivity towards oxidative stress due to H2O2, stress due to the cell wall perturbing agent, SDS, heavy metal stress induced by CuSO4 and moderate sensitivity to high temperatures. Interestingly, deleting HST6 did not influence resistance to LiCl or ZnSO4 (data not shown), but induced resistance to 15 mM FeCl3 compared to the wild type strain. Although no general stress response pathway has been defined in C. albicans (Enjalbert et al., 2003), our findings suggest that Hst6p plays an important role in protecting C. albicans against a range of stress-inducing conditions. On most of the stressors tested, the wild type and mutant strains responded by forming two colony morphological types, i.e. the rough and smooth colony morphology (Fig. 2 on Sucrose, H2O2, SDS and FeCl3). A possible explanation for this may be the fact that C. albicans strain used in this study is capable of switching between these types of colonies on YPD agar, a switching system that is described elsewhere (Chapter IV). Due to this difference in colony types, colony sizes also appear to influence the appearance of spots observed across dilutions on agar plates such that spots with fewer colonies appear large as those with more colonies. However, this discrepancy was counteracted by duplicating each stress experiment. Although ΔHst6 could grow to the same extent on solid media containing 40 mM GSNO, the mutant formed wrinkled colonies with filamentous borders which displayed hyphae when visualized with the light microscope while the wild type strain formed smooth colonies whose cells remained in the yeast phase (Fig. 3). This suggested that Hst6p may play a role in morphological transitions in the presence of nitrogen compounds.
Fig. 2. The role played by Hst6p in response to stress-inducing conditions. Stress-inducing chemicals were supplemented into yeast-extract peptone dextrose agar medium on which serial dilutions of actively growing cells were spotted.

Fig. 3. Response to nitrosative stress on yeast-extract peptone dextrose medium supplemented with 40 mM S-Nitrosoglutathione. LM, light microscopy; Scale bar = 10 µm.

3.2 Colony morphology in response to different nitrogen sources

Since amino acids can initiate morphogenesis and agar invasiveness in C. albicans (Lee et al., 1985), and to further investigate the possible role of Hst6p in nitrogen source induced morphological transition, growth in the presence of different amino
acids was examined on minimal media supplemented with L-amino acids, alanine, leucine, iso-leucine, lysine, methionine, proline, ornithine and threonine in the place of ammonium sulphate [(NH₄)₂SO₄]. Colony and cellular morphology was analyzed at 25 °C, 30 °C and 37 °C. During nitrogen starvation such as growth in the presence of 100 µM amino acids, ammonium permeases, Mep1p and Mep2p are activated following bioavailability of amino acids or ammonium. The highly expressed Mep2p initiates yeast to hyphal transition via the MAP kinase and cAMP-dependent signaling pathways (Biswas & Morschhäuser, 2005). MEP2-mediated yeast-hyphal transition is inhibited when 10 to 100 mM ammonium is added to the medium that contain amino acids as nitrogen sources (Biswas & Morschhäuser, 2005), suggesting that yeast-hyphal morphogenesis may be suppressed by higher nitrogen source availability. After culturing the wild type strain and Δhst6 in the presence of 100 µM amino acids we observed no obvious differences in colony border filamentation at 25 °C, 30 °C and 37 °C (results not shown). Interestingly, in the presence of 10 mM amino acids, filamentous growth at colony borders was observed on all tested amino acids for Δhst6, but not the wild type strain (Fig. 4A). Colonies of the wild type strain only underwent slight filamentation in the presence of ammonium [(NH₄)₂SO₄], L-ornithine and L-proline (Fig. 4A). These data suggested that Hst6p plays a role in suppressing filamentous growth in the presence of an excess (10 mM) nitrogen source. Whether Hst6p hyphal suppression results from activation of transcriptional regulators involved in hyphal repression is yet to be determined.

In the post-wash experiment i.e. after washing off the part of the colony that did not invade the agar surface (Fig. 4B), at 25 °C, the wild type strain appeared to be less invasive when supplemented with amino acids than with (NH₄)₂SO₄ as a sole nitrogen source, except in the presence of L-methionine, where no difference was observed. In accordance with the observed colony fringe filamentation, Δhst6 was more invasive than the wild type strain on all amino acids (Fig. 4B). Leucine, isoleucine, methionine and threonine are classified as nonpreferred amino acids (Ljungdahl & Daiggnan-Fornier, 2012) and these were inhibitory to agar invasiveness in the wild type strain but, interestingly, not towards the invasive phenotype of Δhst6 (Fig. 4B). Similar observations were made after growth at 30 °C (results not shown) although with relatively less pronounced differences between the wild type and mutant strains. No differences were observed in terms of the invasive or colony fringe phenotype between the wild type and isolates of Δhst6 at 37 °C (results not shown).
3.3 Cellular morphology produced in response to different nitrogen sources

We then determined cellular morphology of colonies on nitrogen source plates. This analysis was based on cells that did not penetrate the agar surface i.e. those that formed top colonies as opposed to invasive filaments. As expected, in the presence of 100 µM amino acids the wild type strain and ∆∆hst6 strains were all filamentous at 25 °C, 30 °C and 37 °C (results not shown), possibly due to expression of amino acid permeases. Consistent with our findings on colony border filamentation, ∆∆hst6 cells underwent excessive filamentous growth in response to 10 mM nitrogen sources represented by eight amino acids (Fig. 5A). The wild type strain formed yeast cells displaying only polarized growth, which indicated an attempt to filament in an excess nitrogen source (Fig. 5A). This suggests that Hst6p plays a role in suppressing yeast-hyphal switching in the presence of high concentrations of these amino acids. Although colonies of ∆∆hst6 formed abundant hyphae at the borders at 25 °C, microscopic examination of non-invasive cells of these colonies revealed what may appear as cell wall damage and the emergence of spheroplast-like structures in the presence of all amino acids in this mutant and in only about 50% of tested amino acids (alanine, lysine, ornithine and proline) in the wild type strain (Fig. 6B). Spheroplasts were previously observed by Balish and Svihla (1966) on cells cultivated on sulphur amino acids, cysteine and methionine following treatment of C. albicans with snail gut enzymes. Interestingly, these authors also demonstrated that cells grown on these amino acids are more sensitive to cell wall damage (Balish & Svihla, 1966). According to our data, it is possible that appearance of these structures may not be limited to growth on cysteine and methionine alone but other amino acids, particularly when some genes coding for transport proteins are inactivated or their proteins not expressed (Fig. 5B). Presence of spheroplasts-like
structures was not as conclusive at 30 °C and as a result, a clear distinction between the wild type and \( \Delta \Delta hst6 \) could not be observed. Since this mutant lacks the ATP binding cassette transport gene, \( HST6 \), which our data also implicates in the protection against certain cell wall perturbing agents, we argued that it is perhaps also defective in the protection against by-products of amino acids (e.g. S-adenosylmethionine from methionine), which may escape from the vacuole and induce damage to the cell wall (Balish & Svhilna, 1966). These data further suggest that Hst6p may protect cells from abnormal intracellular accumulation of amino acids/metabolites. This possible role in detoxification was also suggested by Kitanovic et al., 2005 who observed that a \( C. \text{ albicans} \) mutant with a defect in detoxification expresses \( HST6 \) as an attempt to remedy defects in detoxification.

![Figure 5](image-url)

**Fig. 5.** Analysis of cellular morphology after 14 days on minimal media. A. Morphology of cells, which did not invade the agar surface at 37 °C. B. Analysis of damaged cell walls (arrows) in the presence L-amino acids at 25 °C. Glc (control) represent a plate containing 1% glucose without any nitrogen source. Scale bar = 20 \( \mu m \).
3.4 **Response to hyphal-inducing conditions**

To determine whether the role of Hst6p in hyphal formation was also reflected in liquid media, we cultured the strains in hyphal-inducing liquid media containing high and low concentrations of amino acids as well as media that do not contain amino acids as nitrogen source (Lee et al., 1975; Liu et al., 1994; Ells et al., 2011; Lu et al., 2011). Our results indicated that Hst6p also plays a role in hyphal suppression in liquid media containing high concentrations of amino acids (i.e. YPD with serum and Spider medium) (Fig. 6). As expected, ΔΔ*hst6* strains were also filamentous in the presence of synthetic Lee’s medium supplemented with glucose. Interestingly, addition of N-acetylglucosamine to Lee’s medium suppressed hyphal production in ΔΔ*hst6* compared to the wild type strain, suggesting that an alternative regulation might be present in the presence of this carbon source. As expected, in media with low (RMPI-1640 with PGE2) or no amino acids (M9), deletion of *HST6* did not influence germ tube formation.

![Fig. 6](image.png)

**Fig. 6.** Comparison of hyphal production in the presence of hyphal-inducing liquid media at 37 °C. *Significantly different from the wild type strain (p ≤ 0.01).

4. **Conclusions**

The ability of *Candida albicans* to thrive under stress-inducing sites in the host can be attributed to its various coping mechanisms. These include response to oxidative, nitrosative, cell wall and heavy metal stress, osmotic shock and response to starvation. In this work, we present evidence that reveals possible roles of the protein encoded by *HST6*, a functional homologue of *S. cerevisiae* STE6 gene, which encodes for a protein required for the secretion of the Mfa pheromone (Raymond et al., 1998; Magee et al., 2002). To our knowledge, this is the only biological function assigned to this protein in these two yeasts. Therefore, we presented data that suggest that Hst6p may maintain generic biological functions in the pathogenic yeast, *C. albicans*. We show that it plays an important role in stress response. According to the OmniLog phenotypic microarray data, Hst6p seems to
protect the cells from a wide range of stress inducing conditions. These findings were further confirmed by a conventional phenotypic screen employing a set of experiments that induced oxidative, osmotic, heavy metal, cell wall and heat stress. In these experiments, Δhst6 was found to be sensitive while the wild type was resistant. In S. cerevisiae, several other important ABC transporters (e.g. Mdl1p, Ycf1p, Pdr15p, Pdr18p and Snq2p) localized at the mitochondrial and plasma membranes have already been reported to play regulatory roles in stress response (Servos et al., 1993; Li et al., 1996; Wolfger et al., 1997; Chloupkova et al., 2003). Furthermore, our findings of the response to oxygen peroxide were also consistent with that of the ABC transport protein, MacAB, from Salmonella enterica which has been shown to protect cells from oxidative damage (Bogomolnaya et al., 2013), suggesting that the ABC protein stress response system might be widely conserved.

According to the genome wide gene expression profile conducted in our previous studies, HST6 was significantly up-regulated in the presence of AA (Ells, 2011). Exogenous supplementation of AA in yeasts results in its incorporation in the phospholipid bilayer and causes a predisposition to environmental stress. In the closely related pathogenic yeasts, C. albicans and C. dubliniensis, AA enhances antifungal susceptibility by altering the saturation level and fluidity as well as the ergosterol content of cell membranes (Ells et al., 2009). In addition, growth in the presence of polyunsaturated fatty acids causes oxidative stress and apoptosis in C. albicans and C. dubliniensis (Thibane et al., 2012). It is, therefore, reasonable to argue that up-regulation of HST6 gene was part of a stress response system employed by C. albicans during exposure to AA. Interestingly, the work of Ells (2011) also revealed the significant up-regulation of SNQ, also an ABC transport protein encoding gene. In S. cerevisiae Snq2p plays a protective role against metal ion stress (e.g. Na⁺, Li⁺ and Mn²⁺) as well as oxidative stress-inducing chemicals such as 4-nitroquinoline-N-oxide and methyl-nitro-soguanidine (Haase et al., 1992; Decottignies et al., 1995; Decottignies & Goffeau, 1997; Ververidis et al., 2001). In contrast to Hst6p which belongs to the MDR family, Snq2p belongs to the pleiotropic drug resistance (PDR) family, however, both these proteins localize in the plasma membrane. Although Snq2p has largely been reported to play a protective role against stress, our data suggest that ABC transport proteins may also play a predisposing role to some stresses. We observed that Δhst6 was also resistant to heavy metal stress induced by CuSO₄, but resistant to FeCl₃.

Δhst6 also exhibits enhanced filamentous phenotypes in the presence of several amino acids supplemented as sole nitrogen sources on agar media, suggesting that Hst6p also plays an important role in suppressing hyphal production in the presence of amino acids as sole nitrogen sources and during nitrosative stress. Interestingly, Δhst6 was also more filamentous than the wild type strain in most hyphal-inducing conditions that contain high amino acid content. From this data, it can be concluded that the availability of a nitrogen source is crucial for the regulation of HST6 expression. Moreover, the encoding gene was found to be up-regulated in the presence of 38 mM ammonium sulphate both in the MEP2 complement and mutant strains (Dunkel et al., 2014). Together our data and the gene expression data of Dunkel and colleagues (2014), suggest that Hst6p may play an important role in the negative regulation of yeast to hyphal switching, possibly via activation of transcriptional regulators that suppress this process in the presence of an excess nitrogen source. Alternatively, we propose that since this is a transport protein,
Hst6p may regulate yeast to hyphal switching by excreting compounds required to activate transcriptional regulators that promote hyphal induction. In conclusion, this work has presented evidence that suggest there are two generic functions of the Hst6p in addition to the specialized pheromone secretion; the role in stress response and yeast to hyphal switching.

5. References


Chapter IV – The Amino Acid Sensor, Ssy1p, Controls Morphology and Confers Candida albicans Differentially Responsive to Stress-Inducing Conditions

Abstract

*Candida albicans* interacts with its surroundings via the expression of a panel of membrane-bound sensors. These proteins also play roles in phenotypic switching and virulence. *Candida albicans* amino acid sensor, Ssy1p, can activate amino acid uptake and transport during nitrogen starvation. In addition, this protein plays a vital role in colony morphology and yeast to hyphal transition in which genetic determinants required for these processes are switched on. Consistent with these, ΔΔssy1 was found to be defective in colony wrinkling and hyphal formation in this study. The role of *SSY1* in morphogenesis under nitrogen-rich conditions and stress is not clear in literature. Our data suggests that this gene is involved in the regulation of colony and cellular morphology as well as stress under these conditions. According to phenotypic microarrays (PMs) based on the PM09 plate, ΔΔssy1 was resistant to 10-100 mM ammonium sulphate [(NH₄)₂SO₄] while the wild type strain was sensitive. These are the same conditions known to repress yeast to hyphal transition in *C. albicans*. In addition, ΔΔssy1 was resistant to osmotic stress due to sodium phosphate, sodium chloride and sucrose, and maintained normal cellular morphology under nitrosative stress contrary to the wild type strain. Further, ΔΔssy1 was sensitive to heat stress at 37 °C and 42 °C, sodium benzoate and fluconazole while the wild type strain was resistant. Therefore, we propose that Ssy1p results in a differential response to stress. To our knowledge, the Ssy1p has not yet been implicated unambiguously in stress response or drug tolerance.

**Key words:** Amino acid · Filamentation · Morphogenesis · Nitrogen sensing · Phenotypic microarrays · Stress response
1. Introduction

_Candida albicans_ is a causative agent of asymptomatic and symptomatic fungal diseases in humans. As a commensal organism, it occupies locations in the human body which repress pathogenesis and promote interaction with other non-pathogenic non-fungal species (Kavanaugh et al., 2014; Zaborin et al., 2014). Consistent with this, host locales such as the gut are replete with microbes that re-orientate their surroundings to facilitate commensalism and attenuate virulence (Pande et al., 2013). In addition, the presence of mucus which produces heavily glycosylated proteins called mucins has been shown to be the hallmark of the commensal state because of the ability to repress virulence-associated genes (Kavanaugh et al., 2014). However, the host also presents a dynamic micro-environment which can be hostile and promote pathogenesis in commensal strains residing in these diverse niches. As a result, these strains developed biosystems adapted to navigate through the host dynamic conditions. _Candida albicans_ is known for adapting survival mechanisms such as the ability to alternate between two morphological phases, the yeast and hyphal phase (Biswas et al., 2007; Sudbery, 2011; Soll, 2014). The yeast phase is thought to be less virulent compared to the hyphal phase, due to the fact that it cannot penetrate important host tissues, although cases of bloodstream infections have been cited (Sudbery & Berman, 2004; Sudbery, 2011). However, the yeast cells can disseminate and cross the blood barrier into vital organs where the hyphal mode can be triggered through a panel of signaling pathways. These have been the subject of intensive research since they are central to understanding tissue invasion and penetration (Liu et al., 1994; Stoldt et al., 1997; Braun et al., 1999, 2000; Davis et al., 2000; Ramage et al., 2002; Sudbery & Berman, 2004; Homann et al., 2009; Sudbery, 2011; Huang, 2012; Fox et al., 2014; Lu et al., 2011, 2014).

_In vitro_ and _in vivo_ studies indicate that _C. albicans_ utilizes devoted membrane sensors to communicate with the external surroundings and to acquire nutrients (Bregga et al., 2004; Ljungdahl, 2009; Ljungdahl & Daiggnan-Fornier, 2012; Dunkel et al., 2014). For instance, _C. albicans_ remodels phenotypically in response to nutrient availability such as in the case of rich medium and maintains the yeast phase or respond to nutrient limitation by switching to the hyphal phase in an attempt to extract nutrients from distant locations (Maidan et al., 2005; Biswas & Morschhauser, 2005; Dunkel et al., 2014; Vylkova & Lorenz, 2014). This appears to be the rationale behind using _in vitro_ experiments to understand how specific host niches regulate morphogenesis (Granek & Magwene, 2010). The availability of nitrogen plays an important role in _C. albicans_ genetic and phenotypic remodelling. For instance, nitrogen starvation can trigger expression of ammonium permeases, Mep1p and Mep2p and amino acid permeases via GATA transcription factors (Gln3p and Gat1p) and SPS (Ssy1p, Ptr3p and Ssy15) sensing systems, respectively. A switch from the yeast to the hyphal phase can then be activated to explore the microenvironment for available nitrogen sources (Biswas & Morschhauser, 2005; Biswas et al., 2007; Ljungdahl, 2009; Ljungdahl & Daiggnan-Fornier, 2012; Dunkel et al., 2014). The upstream component of the SPS sensor, Ssy1p, is located in the cell membrane and can intracellularly interact with Ptr3p and Ssy5p which, by endoproteolysis, activates two effector zinc-finger proteins, Stp1p (required for protein and peptide utilization) and Stp2p (required for activation of amino acid permeases) (Ljungdahl, 2009). Both Stp1p and Stp2p are crucial for _C. albicans_ virulence. _In vivo_, ΔΔstp1 is attenuated for virulence (Davis et al., 2011) while ΔΔstp2
is defective in pH regulation and yeast to hyphal transition in an acidic phagosomal environment (Vylikova & Lorenz, 2014). Although it is well understood that nitrogen source limitation facilitates its own uptake via the SPS sensor, it is not clear what roles this sensor plays under nitrogen excess and stress conditions. So far it is known that nitrogen has the potential to diffuse and accumulate abnormally and cause damage when available in excessive amounts (Balish & Svhla, 1966; Chapter III) and that SSY1 is expressed upon availability of a nitrogen source, such as ammonium, even at high concentrations (Dunkel et al., 2014). In this study, we show that Δssy1 maintains normal cellular morphology in the presence of high concentrations of ammonium sulphate while the wild type strain cellular morphology is affected. Our data suggests that Ssy1p plays a crucial role in the regulation of cellular morphology in excess nitrogen. Our data further reveal that deletion of SSY1 results in sensitivity to certain stressors, while conferring resistance to others. Therefore, this study reports on biological functions of SSY1 under nutrient rich and stressful conditions.

2. Materials and methods

2.1 Strains used

*Candida albicans* NRRL Y-27077, isolated from a skin lesion was obtained from the Agricultural Research Services Yeast Culture Collection (http://nrrl.ncuar.usda.gov/) and frozen as glycerol stocks in liquid nitrogen until use. After revival, working cultures were obtained on yeast-extract-peptone-dextrose (YPD) agar (10 g/L yeast extract, 20 g/L peptone and 20 g/L glucose and 20 g/L agar). Deletion of SSY1 was conducted as previously described (Chapter II: section 3 and 4) and the independent Δssy1 isolates, UOFS Y-2885 and UOFS Y-2886, were used in this study.

2.2 Analysis of colony morphology using different carbon sources

According to Brega and co-workers (2004), Δssy1 is defective in colony wrinkling and hyphal formation on solid and liquid media respectively, suggesting that the Ssy1p plays a vital role in these processes. In this study, we utilized two independent isolates of Δssy1 in all the conventional stress assays to validate observed phenotypes. However, since similar results were obtained for the mutant strains, only the results of one isolate are shown. In order to ensure that the Ssy1p also maintains these biological functions in our strain, the wild type strain and Δssy1 strains were examined for the ability to form wrinkled colonies or colonies with filamentous borders on solid cultures supplemented with different carbon sources. Briefly cultures were inoculated from a glycerol stock onto YPD plates and incubated for 5 days at room temperature. Following this, a loopful of cells was inoculated into YPD broth and incubated overnight (12-16h) at 30 °C. Cells were resuspended in and washed twice with phosphate buffered saline (PBS) (Sigma-Aldrich, USA) and resuspended in 5 mL of this buffer. Following this, optical density (OD) was read and cells adjusted to OD$_{600nm}$ of 0.08. A 1x and 5x dilution was spotted onto agar plates composed of 6.7 g/L Yeast Nitrogen Base (YNB) (Difco, USA), 20 g/L of the specific carbon source and 20 g/L of agar. Plates were incubated
for up to 14 days and photos were captured (after 3, 7 and 14 days) with a gel documentation system (Bio-Rad XRS, Quantity One 4.5.0) for analysis of colonies. This experiment was carried out in duplicate. A scale of 1 to 5 was then used to score phenotypes displayed by strains on different carbon sources and here we scored the degree of smoothness, wrinkling and filamentous growth. Average scores were calculated and used as final scores.

2.3 Analysis of germ tube formation

Germ tube formation was conducted in liquid cultures. Briefly, cells from a glycerol stock were cultured in YPD broth for 16 h at 30 °C in roller drums. Cultures were harvested, washed with and resuspended in 5 mL PBS. This suspension was diluted to an OD600 of 0.1 into YPD broth supplemented with 10% Fetal Bovine Serum (Sigma-Aldrich, USA), Spider medium (Liu et al., 1994) and Lee’s medium modified according to Lu and co-workers (2011) supplemented with 1.25% glucose (Glc) or N-acetylglucosamine (GlcNAc), and minimal medium (M9) (64 g/L Na2HPO4·7H2O, 15 g/L KH2PO4, 2.5 g/L NaCl, 5 g/L (NH4)2SO4 and 14 g/L agar) supplemented with 1.25% Glc or GlcNAc. Cells were incubated for 18 h at 37 °C and hyphal production was determined. Cells (1000-1500) from ten random fields generated by a camera from three separate experiments were counted at 400x magnification using a Zeiss Axioplan light microscope. In addition, a germ tube assay was conducted according to Ells and co-workers (2011). Briefly, a loop full of cells, grown on YPD agar plates for 24 h at 30 °C, was diluted to an OD600 of 0.1 in filter sterile RPMI-1640 medium (Sigma-Aldrich, USA) with or without 120 pg/mL prostaglandin E2 (PGE2) (Cayman Chemicals, USA). After incubation at 37 °C for 4 h, 100 to 1500 cells were visually assessed for germ tube formation as previously mentioned.

2.4 High throughput phenotypic microarrays

To conduct phenotypic microarrays (PMs), the wild type strain and ΔΔssy1 (UOFS Y-2885) were cultivated at 30 °C for 24 h on Biolog Universal Growth Agar (BioLog, USA), supplemented with 5% (w/v) sheep blood, and inoculated in the 48x yeast nutrient suspension solution prepared as per manufacturer’s recommendations. Phenotypic microarray analysis was conducted as previously discussed (Chapter III, section 2.2).

2.5 Growth on different nitrogen sources

To test growth on medium containing ammonium as sole nitrogen source, we cultured strains on YPD plates for 24 h. Single colonies were selected, washed twice with PBS, and suspended to OD600 of 0.08 in 1.7g/L YNB without amino acids and ammonium sulphate supplemented with glucose to a final concentration of 1% w/v (Glc-YNB w/o). This suspension was incubated for 48 h at 30 °C shaking at 160 rpm and serially diluted (1:100) and spotted on Glc-YNB w/o and Glc-YNB w/o supplemented with 10 mM, 20 mM, 50 mM and 10 mM (NH4)2SO4 (Sigma-Aldrich,
USA) for 3-7 days. Following this, photos were captured with a gel documentation system for analysis of colonies and cells were analyzed using a light microscope. Cells were also measured using the imaging system (analysis doc version 5) in terms of width and length to determine the differences in cell size between the wild type and mutant strains. In addition, growth on amino acids as sole nitrogen sources was determined. This was conducted by preparing the pre-inoculum as described for growth on carbon sources, incubated at 30 °C in minimal media without a nitrogen source for 48 h for nitrogen starvation and spotted on M9 medium containing 1% glucose without any nitrogen source (control), M9 medium with 1% glucose with (NH₄)₂SO₄ or 10 mM filter sterilized amino acids (L-ornithine, L-lysine, L-alanine, L-proline, L-isoleucine, L-threonine, L-methionine and L-leucine) in the place of (NH₄)₂SO₄. This experiment was carried out in duplicate and all plates were incubated at 30 °C and 37 °C for up to 14 days and photos were captured after 3, 7 and 14 days with a gel documentation system and used to determine colony morphology on agar. Agar invasiveness was determined by washing off cells that resulted in colonies at the top of the agar, with a gentle stream of water. Ability of hyphae to penetrate the agar surface was visualized by generating grey scale images using the gel documentation system.

2.6 Analysis of stress on agar medium

The wild type strain and ΔΔssy1 strains were initially plated onto YPD agar and incubated for 24 h at 30 °C. Cells were then resuspended in and diluted with PBS to an OD₆₀₀nm of 0.08. Cells were serially diluted (1:100), spotted on YPD agar and subjected to various stresses present in YPD agar as indicated in Table 1. For analysis of stress phenotypes on this medium, duplicate plates were incubated at 30 °C (unless indicated differently in Table 1) for 3-7 days after which photos were taken and processed for analysis of colonies with stress related phenotypes. Nitrosative stress was determined by streaking cells (resuspended in PBS) onto duplicate plates containing 40 mM S-Nitrosoglutathione (GSNO) followed by incubation at 30 °C. Colonies were analyzed after three and seven days of incubation.

Table 1

<table>
<thead>
<tr>
<th>Stressor</th>
<th>Inducing condition(s)</th>
<th>References</th>
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<tbody>
<tr>
<td>Temperature</td>
<td>Incubation at 30 °C, 37 °C and 42 °C</td>
<td>Homann et al., 2009, Leach et al., 2011, 2012</td>
</tr>
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<td>Heavy metal</td>
<td>Lithium (300 mM LiCl), Zinc (10 mM ZnSO₄), Copper (15 mM CuSO₄); Iron (15 mM FeCl₃)</td>
<td>Homann et al., 2009</td>
</tr>
<tr>
<td>Cell wall</td>
<td>Caffeine (15 mM)</td>
<td>Homann et al., 2009</td>
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<td></td>
<td>Sodium dodecyl sulphate (SDS, 0.04%)</td>
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<tr>
<td>Oxidative</td>
<td>H₂O₂ (6 mM)</td>
<td>Homann et al., 2009, Leach et al., 2011, 2012</td>
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2.7 Susceptibility to antifungal drugs

We examined the response of the wild type and \( \Delta \Delta \text{ssy1} \) cells against a wide range of drugs on paper discs. Fresh cultures from YPD broth were washed twice with and diluted to an \( \text{OD}_{600\text{nm}} \) of 0.08 in 5 mL PBS and spread (150 µL) on YPD plates. Following this, the disc of each test drug [clotrimazole, fluconazole, ketoconazole, miconazole, fluycytocine, amphotericin B and nystatin (Mast Diagnosis, Mersyside, UK)] was positioned at the centre of the YPD plate such that three agar plates contained one disc of a single test drug. These plates were then incubated for 5 days at 30 °C after which a measurement of a zone of inhibition was recorded in millimetres. We then examined plates which had the significant fungicidal effects by analyzing the morphology of cells distant from and those closer to the disc of the test drug on YPD plates using the light microscope. Nuclear condensation and fragmentation of the same cells were analyzed by 4′, 6-diamidino-2-phenylindole (DAPI, Sigma-Aldrich, USA) staining. Cells treated for 24 h, 48 h and 72 h with the drug, which exhibited significant fungicidal effect at 30 °C, were washed twice with PBS and resuspended in permeabilization solution (0.1% Triton X-100 and 0.1% sodium citrate) for 3 min on ice (Thibane et al., 2012). About 1 µg/mL of DAPI was added to the suspension and incubated in the dark at 37 °C for 20 min. Confocal Laser Scanning Microscopy was used to determine cellular morphology and nuclei position.

2.8 Statistical analysis

All experiments, except PM assays which were not repeated, were performed in triplicate unless stated otherwise. A student \( t \)-test was conducted to determine the statistical significant difference between data sets.

3. Results and discussions

3.1 \( \Delta \Delta \text{ssy1} \) cells display alterations in colony and cellular morphology

Ssy1p, along with its downstream components such as Ssy5p, Stp1p and Stp2p, are required for expression of certain virulence factors. As a result, \( \Delta \Delta \text{ssy1} \) is perturbed for colony wrinkling on solid media and hyphal formation in liquid media, phenotypic factors associated with virulence of \( \text{C. albicans} \) (Brega et al., 2004; Homan et al., 2009; Huang, 2012; Vylkova & Lorenz, 2014). To ensure that these phenotypic outputs are maintained in the wild type strain (NRRL Y-27077) used in this study, following deletion of \( \text{SSY1} \), we examined the double deletion mutant strains of this gene for colony and yeast to hyphal morphogenesis. Our data confirms that deleting \( \text{SSY1} \) perturbs expression of these virulence factors in this strain. On solid medium supplemented with different sugars as sole carbon sources, colony borders of \( \Delta \Delta \text{ssy1} \) were not filamentous, with the exception on Spider medium both at 30 °C.
and 37 °C (Fig. 1A). On the basis of colony wrinkling, ΔΔssy1 showed some degree of wrinkling on maltose, sucrose, trehalose, and Spider medium. Interestingly, significant differences were observed in liquid medium in terms of hyphal formation in YPD serum, Spider, Lee’s GlcNAc and minimal media (M9) supplemented with glucose and GlcNAc (Fig. 1B). Similar results were also observed for additional isolates of ΔΔssy1 (results not shown), indicating that Ssy1p still carries its biological roles in C. albicans NRRL Y-27077.

<table>
<thead>
<tr>
<th></th>
<th>25 °C</th>
<th>30 °C</th>
<th>37 °C</th>
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<tbody>
<tr>
<td></td>
<td>Glc</td>
<td>GlcNAc</td>
<td>Mal</td>
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<tr>
<td><strong>WT</strong></td>
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<tr>
<td>ΔΔssy1</td>
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**Symbol** | **Morphology**
---|---
S    | Smooth
W    | Wrinkled
F    | Filamentous/Invasive
Glc  | Glucose
GlcNAc | N-acetyl glucosamine
Mal  | Maltose
Suc  | Sucrose
Spider | Spider Medium
Tre  | Trehalose

**Key:** #1,#2,#4

**Scale**

1

2

3

4

5
Fig. 1. The role of Ssy1p on virulence factors is maintained in C. albicans NRRL Y-27077. A. Colony morphologies reflecting long term hyphal responses between the wild type strain and ΔΔssy1 on different carbon sources after seven to 7 days at 25 °C, 30 °C and 37 °C. B. Short term hyphal responses using known hyphal-inducing media at 37 °C after 18 h.

3.2 SSY1 plays a role in controlling morphology under conditions replete with ammonium and amino acids

We applied phenotypic microarrays (PMs) to screen for the ability of the wild type strain and ΔΔssy1 to respond to a wide variety of culture conditions. This is due to the ability of this system to generate high throughput data based on tetrazolium dye reduction as a reporter system. During incubation, PM microplates are photographed every 15 minutes, capturing the intensity of colour as tetrazolium is reduced to formazan and resulting in standard curves reflecting respiring cells (Homann et al., 2005). According to this analysis, the most significant differences were observed in the PM09 plate containing stress-inducing substrates. As indicated in Fig. 2A, it was observed from this data that deleting SSY1 conferred tolerance to conditions with high ammonium content. ΔΔssy1 displayed higher dye reduction, indicative of more cells respiring in response to 10 mM, 20 mM, 50 mM and 100 mM ammonium sulphate ((NH₄)₂SO₄) while the wild type strain displayed poor reduction, indicative of high sensitive to these conditions. This data suggests that SSY1 results in sensitivity to conditions replete with ammonium. Since PM assays were not repeated, we validated this data by culturing both the wild type strain and ΔΔssy1 (including an additional isolate) on agar plates without and with up to 100 mM (NH₄)₂SO₄. While the wild type and ΔΔssy1 strains showed no differences in colony and cellular morphology after 3-7 days on the agar plates lacking (NH₄)₂SO₄, there was a dramatic difference when this nitrogen source was added (data is presented in Fig. 2B). The wild type strain formed smooth colonies on all (NH₄)₂SO₄ concentrations while ΔΔssy1 formed increasingly rough and wrinkled colonies with
an increase in (NH$_4$)$_2$SO$_4$ concentration at 30 °C. The differences of colony morphology between these two strains were not as obvious at 37 °C (results not shown) as they were at 30 °C. When cellular morphology at 30 °C was analyzed, the wild type strain displayed large and smaller cells which were round in most cases while cells of ΔΔssy1 were mostly elongated (Fig. 2C). When we measured the size of the cells from the wild type and mutant strain using the accompanying light microscope software, the wild type strain cells were relatively larger while ΔΔssy1 cells were comparatively smaller on all (NH$_4$)$_2$SO$_4$ concentrations (Table S1). Similar cell sizes were observed at 37 °C (results not shown). According to Biswas and Morschhauser (2005) and Dunkel and co-workers (2014), nitrogen starvation (100 µM) activates expression of ammonium and amino acid permeases and ammonium uptake and thereafter filamentation. Supplementing 10 mM ammonium to these starvation conditions represses filamentation by repressing permeases possibly by activating their endocytosis into proteosomes and ultimately their degradation. Likewise, we have previously demonstrated that 10 mM amino acids supplemented as sole nitrogen sources can repress filamentation (Chapter III), possibly due to repression of amino acid permeases by repressing amino acid sensing. Interestingly, we observed that under similar culture conditions i.e. 10 mM ammonium and amino acids, ΔΔssy1 was more invasive than the wild type strain on some amino acids (Fig. 2D) at 25 °C and 30 °C while the degree of invasiveness was the same for both strains at 37 °C.
C

<table>
<thead>
<tr>
<th>Concentration</th>
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**Fig. 2.** The role of Ssy1p under conditions replete with ammonium and amino acids. A. Phenotypic microarrays (PMs) showing response towards ammonium sulphate [(NH₄)₂SO₄] as a source of ammonium after 48h at 30 °C. B. Colony morphology on yeast nitrogen base without amino acids and (NH₄)₂SO₄ after 3 days at 30 °C. 0 mM (NH₄)₂SO₄ represents a plate which contains only glucose and no nitrogen source (control) C. Cellular morphology on similar conditions as in B. Scale bar represents 20 µm and 10 µm at 400x and 100x respectively. D. Agar invasiveness after 14 days at 25°C, 30 °C and 37 °C on YNB w/o supplemented with 10 mM (NH₄)₂SO₄ and 10 mM amino acids. Blocks with broken edges are indicative of increased agar invasiveness in ΔΔssy1.

3.3 *Deleting SSY1 causes a differential response to stress-inducing conditions*

According to the OmniLog phenotypic microarray results, the ΔΔssy1 strain exhibited some degree of resistance towards a range of substrates when compared to the wild type in the PM09 plate as indicated in Fig. 4A. These included relative resistance to inorganic salts, sodium sulphate, sodium phosphate, sodium formate, sodium lactate and 20 mM sodium benzoate as well as organic compounds such as ethylglycol and urea. Interestingly, ΔΔssy1 was more sensitive to NaCl concentrations above 3% than the wild type, even when various osmoprotectants were added. Although the wild type strain was sensitive to sodium nitrate after 24 h (Fig. S1), it grew to a similar extent when compared to ΔΔssy1 in response to this compound after 48 h. Interestingly, the wild type strain was sensitive while ΔΔssy1 was resistant to increasing concentrations of sodium nitrite, known to induce nitrosative damage in *C. albicans* (Ullmann et al., 2004; Chiranand et al., 2008). This phenotypic microarray data suggests that Ssy1p results in a differential response to different stresses since deletion of *SSY1* provides tolerance to certain stress-inducing conditions while resulting in susceptibility to others. To further analyse this role, we cultured the wild type and mutant strains in the presence of stress-inducing
conditions for 3-7 days and analyzed colonies for stress related phenotypes (Fig. 4B). According to this conventional screen, \( \Delta \Delta ssy1 \) became sensitive to heat stress at 37 °C and 42 °C. To our surprise, the wild type strain was sensitive while \( \Delta \Delta ssy1 \) was resistant to osmotic stress due to sodium chloride, although the opposite response was observed with the PM assays. Response to osmotic stress was confirmed with the disaccharide sugar, sucrose, and using two independent isolates of \( \Delta ssy1 \), suggesting that indeed deleting \( SSY1 \) confers tolerance to osmotic conditions. We reason that these discrepancies in the high throughput and conventional screens may point to deviations in culture dynamics between these phenotypic screens. In addition, the wild type strain was sensitive while \( \Delta \Delta ssy1 \) remained resistant to cell wall stress due to caffeine and heavy metal stress due to iron and lithium chloride. In terms of nitrosative stress due to GSNO, the effect of GSNO on both the wild type strain and \( \Delta \Delta ssy1 \) was less reflected in colony morphology and growth; both the wild type and isolates of \( \Delta ssy1 \) formed smooth colonies after 3 days on YPD GSNO as indicated in Fig. 4C. Interestingly, the effect of GSNO was reflected in morphology of the wild type cells which became enlarged, with large vacuoles. Nonetheless, \( \Delta \Delta ssy1 \) displayed an elongated cellular shape with no signs of inflation in response to GSNO, suggesting that it was experiencing little if any nitrosative stress. This data suggests that although NO does not exert cidal effects on the wild type cells (Brown et al., 2014), it has a major influence on cellular morphology. Further, this data is consistent with responses towards conditions replete with \((NH_4)_2SO_4\) and response to sodium nitrite observed for the wild type and mutant strains (section 3.2).
Fig. 3. The role of Ssy1p in stress-inducing conditions. A. High throughput phenotypic microarrays showing a differential response of ΔΔssy1 to stressful conditions in the PM09 plate. Dashed and solid boxes indicate compounds to which ΔΔssy1 was sensitive and resistant respectively. B. Conventional screening using yeast-extract peptone dextrose agar. Colonies were analyzed for stress related phenotypes after 3-7 days at 30 °C. C. Colony and cellular morphology after exposure to nitrosative stress-inducing conditions after 3-7 days. Scale bar represents 10 µm.

3.4 SSY1 plays a role in adaptation to antifungal treatment

We determined the response of ΔΔssy1 to other stresses such as exposure to antifungal drugs by subjecting the wild type and mutant strains to the most common antifungal drugs. These were amphotericin B, flucytocine, nystatin and azoles i.e. clotrimazole, fluconazole, ketoconazole and miconazole. Out of all these antifungal drugs, only fluconazole (FLC) caused a significant change in phenotype (Fig. 5A: YPD culture). Fluconazole is known to inhibit the fungal cytochrome P450 enzyme, CYP51A1 (lanosterol 14 α-demethylase), required for the synthesis of the major fungal sterol, ergosterol, from lanosterol (White et al., 1998), resulting in membrane permeability. We continued our analyses based on thisazole drug. Fluconazole, which specifically targets Erg11p, is the most prescribed antifungal drug in patients with fungal infections (White et al., 1998). However, some clinical isolates of C. albicans display resistant to this drug due to mutations in the ERG11 gene (White et al., 1998; Xiang et al., 2013). Furthermore, about 50% of FLC resistant isolates are aneuploid and these aneuploidies can also confer FLC resistance (Selmecki et al., 2005, 2006, 2008, 2009). A recent report (Harrison et al., 2014) demonstrates that aneuploidies in resistant isolates are preceded by abnormally large tetraploid intermediates (multinucleate connected cells) with increased DNA content called trimeras (two buds that fail to separate) or multimeras (long chain of buds that failed to separate). Our drug susceptibility experiments are consistent with these findings. Candida albicans wild type strain used in this study is resistant to FLC and at high
concentrations (i.e. near the disc of the test drug) forms very small colonies (Fig. 4A). When these colonies were analyzed by light microscopy, structures that were reminiscent of trimeras and multimeras were observed (Fig. 4A). In contrast, FLC was fungicidal to ΔΔssy1 (zone of inhibition: 40.33 ±5.51 mm). Cells of the mutant strain taken from small colonies i.e. those near the FLC test disc, although present in very small numbers due to cidal effects, formed little or no trimeras (Fig. 4A). Cells from the wild type strain and ΔΔssy1 were also analyzed using DAPI staining to examine their nuclei positions. From this data, it was confirmed that trimeras and multimeras possess abnormal nuclear divisions which explains the observed tetraploid intermediates and increased genetic material (Fig. 4B) (Harrison et al., 2014). Interestingly, ΔΔssy1 formed cells characterized by normal nuclear division (Fig. 4B), suggesting that the Ssy1p plays an important role in aneuploid formation and henceforth response to antifungal drugs.

**Fig. 4.** The role of Ssy1p in response to fluconazole (FLC) and tetraploid intermediates. A. Colony and cellular morphology after exposure to FLC after 3 days at 30 °C. Below plate cultures are micrographs showing cellular morphology distant from (↓FLC) and near the disc (↑FLC) mounted with the test drug. Scale bar represents 10 μm. B. Confocal Laser Scanning Microscopy of cells scraped from ↑FLC after 24h, 48h and 72h. Scale bar represents 10 μm.
4. Conclusions

*Candida albicans* resides in the human host in several forms. It can reside as a commensal organism which is harmless to the host or switch to the pathogenic phase and inflict harm when the host immune response is compromised. Accordingly, *C. albicans* interacts with and explores the human micro-environment using a panel of devoted membrane sensors which can be integrated into the plasma membrane. In this study, we implicate the amino acid sensor, Ssy1p, in stress adaptation. We first show that *SSY1* maintains its biological functions in colony wrinkling and hyphal formation in *C. albicans* strain, NRRL Y-27077. We also utilized PMs to screen for phenotypes with stress related properties. Analysis based on this screen suggests that Ssy1p plays an important role in stress. However, this technology, as powerful as it appears to be, has some setbacks. For instance, not all PM plates include necessary controls such as the PM09 plate used in this study. As a result, repetition with complemented strains or additional mutant strains (independent isolates) becomes a challenge. Therefore, some of the data in this study had to be validated, with proper controls, with conventional screens using independent isolates of the mutant strain. Some authors also validated PM assays with conventional screens to confirm phenotypic outputs from deleterious effects (Homann et al., 2005). With conventional screens, we uncovered that Ssy1p confers *C. albicans* differentially responsive to a wide range of stressful conditions. For instance, while ΔΔssy1 is tolerant to osmotic, cell wall and heavy metal, it was also found to be sensitive to high temperatures in comparison to the wild type counterpart. Interestingly, our data also highlights the role of Ssy1p in conditions rich in nitrogen. In contrast to the wild type strain, ΔΔssy1 was resistant to up to 100 mM (NH₄)₂SO₄ according PMs. On agar medium, the mutant strain displayed colony and cellular morphology different from the wild type strain, suggesting that *SSY1* is involved in regulating morphology under nitrogen-rich conditions. This is consistent with the fact that this gene also regulates hyphal morphogenesis in *C. albicans*. Further, ΔΔssy1 strains were susceptible to FLC and failed to form tri- and multimeras when exposed to this antifungal drug in comparison to the wild type strain, suggesting that *SSY1* plays an important role in resistance to antifungal therapy. With the dire need for novel antifungal targets, Ssy1p should be investigated as a possible drug target to combat *C. albicans* infections.

According to the genome wide gene expression profile generated in our research group (Ells, 2011), *SSY1* was one of the genes found to be significantly upregulated in the presence of a polyunsaturated omega-6 fatty acid 20:4, arachidonic acid (AA), after 8 h, 24 h and 48 h. Arachidonic acid does not form part of the phospholipid bilayer of pathogenic yeasts although these microorganisms can convert it to immunomodulatory eicosanoids such as prostaglandin E₂ (PGE₂) (Erb-Downward & Noverr, 2007; Erb-Downward et al., 2008; Brodhun & Feussner, 2011; Ells et al., 2012). In the pathogenic yeasts, *C. albicans* and *C. dubliniensis*, AA enhances antifungal susceptibility by disturbing the saturation level and fluidity as well as the ergosterol content of cell membranes (Ells et al., 2009). Since our phenotypic data implicates *SSY1* in a differential response to stress conditions, it is possible that this gene was up-regulated in the presence AA to respond to stress.
5. References


Supporting information
Table S1

Measured cell sizes of the wild type strain and ΔΔssy1 (UOFS Y-2885) at 30 °C after 3 days on yeast nitrogen base without amino acids and ammonium sulphate ((NH₄)₂SO₄) supplemented with increasing concentrations of (NH₄)₂SO₄. L – length, W – width.

<table>
<thead>
<tr>
<th>(NH₄)₂SO₄</th>
<th>Cell size</th>
<th>ΔΔssy1</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>WT (µm)</td>
<td>ΔΔssy1 (µm)</td>
</tr>
<tr>
<td>0 mM</td>
<td>2.2 µm – 4.8 µm</td>
<td>2.6 µm – 5.4 µm</td>
</tr>
<tr>
<td></td>
<td>1.2 µm – 4.7 µm</td>
<td>1.5 µm – 3.7 µm</td>
</tr>
<tr>
<td>10 mM</td>
<td>3.2 µm – 5.9 µm</td>
<td>2 µm – 6.1 µm</td>
</tr>
<tr>
<td></td>
<td>2.2 µm – 3.6 µm</td>
<td>0.7 µm – 2.7 µm</td>
</tr>
<tr>
<td>20 mM</td>
<td>2.3 µm – 5.7 µm</td>
<td>2.5 µm – 6.9 µm</td>
</tr>
<tr>
<td></td>
<td>1.6 µm – 3.2 µm</td>
<td>0.9 µm – 2.6 µm</td>
</tr>
<tr>
<td>50 mM</td>
<td>3.6 µm – 9.3 µm</td>
<td>2.7 µm – 6.2 µm</td>
</tr>
<tr>
<td></td>
<td>1.8 µm – 3.9 µm</td>
<td>0.5 µm – 1.7 µm</td>
</tr>
<tr>
<td>100 mM</td>
<td>2.3 µm – 5.5 µm</td>
<td>2.9 µm – 6.6 µm</td>
</tr>
<tr>
<td></td>
<td>1.6 µm – 3.4 µm</td>
<td>0.8 µm – 1.8 µm</td>
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Fig. S1. Response to sodium nitrate and sodium nitrite after 24 h at 30 °C.
Chapter V – Characterization of the “White-Gray-Opaque-Like” Switching System (and its Effects on Virulence Factors) and ORF19.4612

(Submitted for review in the journal, Fungal Genetics and Biology; work presented follows the format of this journal)

1. Abstract

Host adaptation in the human fungal pathogen, *Candida albicans*, is achieved by generating suitable phenotypes via morphological switching. White-opaque switching is one of the most widely analyzed morphological switching systems that generate mating competent cells under favourable conditions. This system has been dubbed the white-gray-opaque switching after the discovery of the gray phenotype. In this study, we describe the “white-gray-opaque-like” (WGOL) switching system comprising of three unique phenotypes termed light (LP), dark (DP) and rough pink (RP) based on phloxine B staining, in addition to the white phenotype. These resemble and differ from opaque, GUT and gray phenotypes in important aspects, such as cell surface pimples as well as colony colour and texture. Furthermore, LP, DP and RP cells can form hyphae and biofilms under conditions that induce white cell filamentation, although some are influenced by temperature. These biofilms can produce PGE$_2$ that are, in some cases, temperature dependent. In addition, differences in fluconazole susceptibility of biofilms produced by the different cell types were observed. Based on our results, we propose that the WGOL switching is a previously undescribed system that might contribute to host adaptation, commensalism and pathogenesis in *C. albicans*. In addition, we report on the characterization of ORF19.412, a previously uncharacterized gene playing an important role in the WGOL switching, filamentation and PGE$_2$ production. For instance, Δorf19.4612 fails to form RP colonies normally seen in the wild type cultures while the wild type RP cells produce more PGE$_2$ at 37 °C. Moreover, contrary to the wild type RP cells, ΔOrf19.4612 RP cells produce considerably more PGE$_2$ at 37 °C than at 25 °C. Further, ΔOrf19.4612 is more filamentous than the wild type strain on most culture conditions.

**Keywords:** Biofilms · Pathogenesis · Prostaglandin E$_2$ · Phenotype · Transition
2. Introduction

The human fungal pathogen, *Candida albicans*, can thrive in a mammalian host by generating varying morphological phases (Biswas et al., 2007; Soll, 2014). Morphological switching is considered a central virulence factor contributing to infections in immunocompromised and immunosuppressed patients. One of the most widely analyzed morphological switching systems in *C. albicans* is white-opaque switching which results in mating competent opaque cells under favourable conditions. Cells in the white phase are relatively round and their colonies appear dome-shaped, while cells in the opaque phase are large and elongated and their colonies are flat and appear dark pink on phloxine B plates (Soll, 1997, 2014). White cells express white-specific genes and follow a general filamentation programme (Biswas et al., 2007), while opaque cells express opaque-specific genes (Soll, 1997) and follow a specialized program of filamentation (Si et al., 2013). In addition, the transcription factor, *EFG1*, is the main regulator of white cell filamentation (Stoldt et al., 1997; Biswas et al., 2007; Huang, 2012) while *BCR1*, the main biofilm regulator in white cells (Nobile & Mitchell, 2005), regulates opaque cell filamentation (Guan et al., 2013). Both white and opaque cell types are heritable over a number of generations. White-opaque transition is initiated by the mating type locus (*MTL*), making way for the transcriptional network mainly controlled by Wor1p and Efg1p. The *MTL* locus also regulates mating between *C. albicans* a and α strains (Miller & Johnson, 2002). The a1 and a2 proteins, encoded at the *MTL* locus, form a complex and repress Wor1p, resulting in the normal white phase observed in many clinical isolates (Zordan et al., 2006, 2007). Therefore, strains that are capable of switching between the two phases are expected to be homozygous at the *MTL* locus (Lockhart et al., 2002), however, there is no evidence suggesting that all *MTL* homozygous strains are capable of switching between white and opaque (Lockhart et al., 2002). Interestingly, N-acetylglucosamine and 5% carbon dioxide (CO₂) can induce switching in some natural isolates that are heterozygous at the *MTL* locus despite their inability to undergo mating (Xie et al., 2013).

Ramírez-Zavala and co-workers (2008) discovered that passaging *MTLa* white cells into the mammalian gastrointestinal tract triggers white-opaque switching. Recently, Pande and co-workers (2013) discovered that passaging *MTLa/α* induces the formation of an opaque-like phenotype, referred to as the gastrointestinal transition (GUT) phenotype. GUT cells bear a close resemblance to opaque cells except that they lack surface pimples. Although only induced by the mammalian gastrointestinal tract, GUT cells are stable at 30 °C and 37 °C in vitro provided that the master regulator of opaque cell formation, Wor1p, is overexpressed (Pande et al., 2013). Furthermore, as opposed to opaque cells which exist as either *MTLa* or *MTLα*, GUT cells are *MTLa/α* (Pande et al., 2013). In addition to the GUT cells, Tao and co-workers (2014a) discovered a phenotype on rich, yeast-extract peptone dextrose (YPD) medium, which is distinct from the traditional white and opaque, as well as the GUT cells and referred to it as the “gray” phenotype. The distinction in the gray cells from white and opaque cells was observed in their mating efficiency, which is higher than the white cells but lower than the opaque cells, and production of secreted aspartyl proteinases (SAPs), which was found to be significantly higher in the gray cells compared to the white and opaque cells (Tao et al., 2014a). In addition, gray cells appear pink on YPD supplemented with phloxine B, are smaller than opaque cells and lack obvious surface pimples. Although the global gene expression profiles of gray cells overlapped with that of white and opaque cells,
genes such as HSP31, LIP1, CAS2 and PGA26 were highly enriched, while ORF19.6200 and ORF19.6070, we exclusively enriched in these cells (Tao et al., 2014a).

In this study, we describe unique forms of colony phenotypes that resemble the white-gray-opaque tristable transition observed from a C. albicans strain isolated from a skin lesion. Due to this resemblance, we refer to this set of phenotypes as the white-gray-opaque-like (WGOL) system. We examine the role of various environmental conditions on this switching system to affect the ability to generate different cell types and to form virulence factors such as germ tubes, biofilms and prostaglandin E₂ (PGE₂). We also report on the role of a previously uncharacterized gene, ORF19.4612, in this switching system.

3. Materials and methods

3.1 Strains used

Candida albicans NRRL Y-27077, isolated from a skin lesion was obtained from the Agricultural Research Services Yeast Culture Collection (http://nrrl.ncaur.usda.gov/) and frozen in as glycerol stocks in liquid nitrogen until use. After revival working cultures were obtained on yeast-extract, peptone and dextrose (YPD) agar (10 g/L yeast extract, 20 g/L peptone and 20 g/L glucose and 20 g/L agar). Δorf19.4612 strains used in this study (UOFS Y-2877, UOFS Y-2878, UOFS Y-2895 and UOFS Y-2896) were constructed as described in Chapter II (section 3 and 4.3).

3.2 Growth analysis and phenotypic switching from mixed cultures

Growth analysis was conducted between the wild type and mutant strain to ensure that switching was not influenced by slow or fast growth. Briefly, a loopful of cells from the wild type strain and Δorf19.4612 (UOFS Y-2877, UOFS Y-2878, UOFS Y-2895 and UOFS Y-2896) was inoculated in YPD broth and incubated at 30 °C shaking for 16 h. Following this, cells were adjusted to an OD₆₀₀nm of 0.08 in 10 mL fresh YPD broth in 250 mL Ellen Meyer flaks and incubated in triplicate at 30 °C. Optical density (OD₆₉₀nm) readings were taken every 90 min for 16 h and this data was used to construct growth curves for growth analysis between the wild type and mutant strains. Morphological switching assays were conducted similarly to the white-opaque switching assays described previously (Huang et al., 2010; Tao et al., 2014a). In addition, since several factors are known to influence colony phenotype, (i.e. incubation temperature, incubation time and glucose concentration) the influence of these on the colony phenotype of C. albicans NRRL Y-27077 was assessed using a 2X2X3 factorial design, incorporating two incubation temperatures, two incubation periods and three glucose concentrations in YPD media supplemented with 5 µm/mL phloxine B (Merck, Germany). Cells from the wild type and Δorf19.4612 strains (UOFS Y-2877, UOFS Y-2878 and UOFS Y-2895) were streaked from a 15% glycerol stock onto YPD plates and incubated for 10 days at room temperature for revival, followed by incubation at 30 °C in YPD broth for 24 h before inoculating into the different media. This culture was diluted from OD₆₀₀nm of 0.08 and adjusted to 2×10⁶. This adjusted culture was plated on 0.4%, 2% and 4%
glucose supplemented in YPD (Glc YPD) agar to determine switching from mixed cultures. The percentage of the different colony phenotypes were determined and cellular morphology was examined using a Zeiss Axioplan light microscope.

3.3 Scanning electron microscopy

Scanning electron microscopy was carried according to Van Wyk & Wingfield (1994). Briefly, wild type and Δorf19.4612 (UOFS Y-2877 and UOFS Y-2878) cells (5-10 days old) from YPD agar plates were fixed using 3% v/v of phosphate buffered glutaraldehyde (Sigma-Aldrich, USA) solution at pH 7.0 and a 1% m/v solution of osmium tetroxide (Sigma-Aldrich, USA) for 4 h. Following this, the fixed cells were dehydrated in a series of ethanol solutions (30%, 50%, 70%, 90%, and 100%) for 30 min per solution. The ethanol-dehydrated cells were then critical-point dried, mounted, and spatter coated with gold to achieve conductivity. Scanning electron micrographs were generated with a JEOL 6400 WIN-SEM.

3.4 Secreted aspartyl proteinase activity assay

Activity of SAPs was determined on chemically defined media, Yeast Nitrogen Base without amino acids and ammonium sulphate (Difco, USA) supplemented with 1% w/v glucose (Glc-YNB w/o). Briefly, wild type and Δorf19.4612 cells (UOFS Y-2877 and UOFS Y-2878) were allowed to form different colony phenotypes on YPD agar at 25 °C or 37 °C and incubated for 4 h at 30 °C in Phosphate Buffered Saline (PBS) (Sigma-Aldrich, USA) in order to deplete intracellular nutrients. Cells were then washed five times with PBS and diluted to 5x10^6 cells/mL. Of this standardized cell suspension, 5 µL was spotted on (Glc-YNB w/o) supplemented with 0.2 % Bovine Serum Albumin (BSA) (Roche, USA) as a sole nitrogen source. Development of the white halos around the colonies was monitored over a period of 6 days at 25 °C.

3.5 Switching frequency under different culture conditions

In order to determine the ability of one phenotype to switch to any of the other phenotypes, a colony displaying a specific phenotype from the wild type and Δorf19.4612 strains (UOFS Y-2877, UOFS Y-2878 and UOFS Y-2895) was inoculated into YPD broth, after which the same experimental design as described above was followed. This YPD broth culture was diluted from OD_{600nm} of 0.08 and adjusted to 2x10^6. Plates were incubated at 25 and 37 °C in air or at 37 °C in 5% CO₂ and analyzed after 3 and 5 days for the development of alternative colony phenotypes. Since C. albicans NRRL Y-27077 formed very small colonies on Lee's medium (which made quantification difficult), YNB, supplemented with 1.25 % glucose or N-acetylglicosamine and 5 µm/mL phloxine B was used for switching on chemically defined medium and incubated in air and 5% CO₂ at 37 °C for 5 days. Micrographs were obtained and cellular morphology was examined using a Zeiss Axioplan light microscope.
3.6 Germ tube and hyphal production assays

Wild type and Δorf19.4612 (UOFS Y-2877 and UOFS Y-2878) cells were cultured in YPD broth for 16 h at 30 °C and harvested by centrifugation. This was followed by washing steps with and resuspension in 5 mL PBS. This suspension was diluted to an OD_{600nm} of 0.5 in YPD and RPMI-1640 (Sigma-Aldrich, USA) supplemented with 10% Fetal Bovine Serum (Sigma-Aldrich, USA), Lee’s medium (Lee et al., 1975), modified according to Lu and colleagues (2011), supplemented with 1.25% glucose or N-acetylglucosamine as well as Spider medium (Liu et al., 1994). Cultures were incubated at 37 °C with shaking at 160 rpm for 4 h (short term hyphal response). Approximately 500 cells from triplicate samples were analyzed for germ tube production with a light microscope at 400x magnification. To test for filamentous growth on solid media (long term hyphal responses), we cultured strains on YPD plates for 24 h. Single colonies were selected, washed twice with PBS, and suspended in 5 mL of this buffer and incubated at 30 °C in minimal media without a nitrogen source for 48 h for nitrogen starvation and spotted on M9 medium containing 1% w/v glucose without any nitrogen source (control), 1% glucose with (NH₄)₂SO₄ or 10 mM filter sterilized amino acids (L-ornithine, L-lysine, L-alanine, L-proline, L-isoleucine, L-threonine, L-methionine and L-leucine) in the place of (NH₄)₂SO₄. This experiment was carried out in duplicate and all plates were incubated at 25 °C, 30 °C and 37 °C for up to 14 days. Photos were captured after 7 and 14 days with a gel documentation system and used to determine colony morphology on agar. Agar invasiveness was determined by washing off cells at the top of the agar with a gentle stream of water. Ability of hyphae to penetrate the agar surface was visualized by generating grey scale images using the gel documentation system.

3.7 Biofilm formation and viability

Five day old YPD agar cultures from the wild type and Δorf19.4612 (UOFS Y-2877 and UOFS Y-2878) were used to form biofilms from different cell types due to the fact that after this period cells display distinct colony morphologies. A loopful of cells from each cell type was resuspended in 5 mL PBS contained in 15 mL polypropylene tubes (Becton–Dickinson Labware, USA) and incubated for 4 h at 30 °C to deplete intracellularly remaining YPD nutrients. Cells were harvested by centrifugation, washed twice with PBS and resuspended in 20 mL filter sterilized RPMI-1640 medium to a final concentration of 1x10⁶ cells/mL. These suspensions were transferred into 96-well microtitter plates (Costar, USA) and incubated at 25 °C and 37 °C for 48 h to allow formation of mature biofilms. Biofilm cell viability was determined as previously described (Ells et al., 2011). Briefly, the medium was removed by aspiration and plates washed twice with 200 µL PBS to remove non-adherent cells. After this, 50µL of XTT(2,3-bis(2-methoxy-4-nitro-5-sulfophenyl)-5[(phenylamino) carbonyl]-2H tetrazolium hydroxide (XTT) (Sigma–Aldrich, USA) (1g XTT in 1L Ringer’s lactate solution, filter sterilized, aliquoted and stored at -80 °C) and 4µL of 1mM menadione (10mM menadione in acetone which was diluted to a final 1mM concentration) was added to each well and plates were incubated for 3 h in the dark at 37 °C. The absorbance from each well was measured at 492 nm.
3.8 Fluconazole sensitivity assay
For drug sensitivity experiments, biofilms from the wild type and Δorf19.4612 strains (UOFS Y-2877 and UOFS Y-2878) were prepared in RPMI-1640 medium as previously described. The medium was supplemented with 150 µg/mL fluconazole (FLC) (Fluka). Medium with only cells was used as positive control, whereas medium without the cells was used as a negative control. The plates were incubated at 25 °C and 37 °C for 48 h after which biofilm viability was measured using XTT as previously described.

3.9 PGE$_2$ production from C. albicans biofilms
To measure PGE$_2$ production by different cell types, colonies from the wild type and Δorf19.4612 strains (UOFS Y-2877 and UOFS Y-2878) representing different phenotypes on YPD agar supplemented with phloxine B were selected, washed twice with 5 mL PBS, resuspended in 10 mL of this buffer and incubated for 4 h at 30 °C to deplete intracellular YPD nutrients. Cells were counted and diluted to $1 \times 10^6$ cells/mL in 20 mL RPMI-1640 medium containing a final concentration of 500 µM arachidonic acid (AA) (Sigma-Aldrich, USA) in 0.38% (v/v) ethanol. These suspensions were dispensed into 90mm polystyrene Petri dishes (Merck, Germany) and incubated at 25 °C and 37 °C for 48 h to allow formation of mature biofilms (Ells et al., 2011). The supernatant was removed and filtered through 0.2 µm cellulose acetate syringe filters. Prostaglandin E$_2$ was extracted from the supernatant using 15 mL ethyl acetate (Merck, Germany) (Erb-Downward & Noverr, 2007). The organic phase was removed and evaporated with nitrogen gas at room temperature. Samples were resuspended in absolute ethanol and analyzed using an API3200QTRAP hybrid triple quadrupole linear ion trap mass spectrometer (ABSciex, Germany) with an Agilent 1,200 SL series HPLC front end. Briefly, samples were injected at a flow rate of 500 µL/min at 30 °C. Sample separation was carried out with Zorbax Eclipse XDB C18, 50 x 4.6 mm column (Agilent Technologies, Germany) consisting of mobile phase of 10 mM ammonium formate aqueous solution in 5% methanol and in 95% methanol. Commercial PGE$_2$ was used as a reference standard and was infused into the instrument while compound optimization feature, included in Analyst™ software, was used to build a five transition (one precursor producing five unique fragments) multiple reaction monitoring method prior to sample separation. Prostaglandin E$_2$ was determined from five recorded transitions (351.2/315.2; 351.2/271.2; 351.2/333.3; 351.2/189.0 and 351.2/235.1) selected for the final LCMS/MS method. To determine biofilm biomass, biofilms were washed with PBS, scraped off the Petri dishes and filtered through pre-weighed 0.2 µm cellulose acetate filters. Filters containing biofilms were dried to constant weight for 24 h at 37 °C after which the biomass was determined.

3.10 Statistical analysis
All experiments were performed in a least in triplicate unless stated otherwise. A student $t$-test was conducted to determine the statistical significant difference between data sets. A $p$ value of $\leq 0.05$ was considered significant.
4. Results and discussion

4.1 Discovery of the “white-gray-opaque-like” morphological phenotypes

We observed that *C. albicans* NRRL Y-27077 forms colonies that resemble white and opaque colonies when grown on YPD agar at 25 °C (Fig. S1). Interestingly, when this strain was cultured on YPD supplemented with phloxine B, more than the expected two phenotypes were observed. These were white (Wh) (Fig. 1A), smooth light pink (LP) (Fig. 1B), smooth dark pink (DP) which formed when glucose concentration was increased to 4% (Fig. 1C) and rough pink (RP) (Fig. 1D). Using microscopic examination, we observed that cells of the white colonies have a usual round to oval shape (Fig. 1F) while those of the smooth LP colonies are elongated (Fig. 1G). Cells of DP colonies are elongated but mostly appear larger when compared to white and LP cells (Fig. 1H). Interestingly, the RP phenotype also comprised of larger cells although there were occasionally smaller cells observed (Fig. 1I). But perhaps the most distinguishing character of this phenotype was the slight abnormal shape of cells when compared to other cell types.

![Fig. 1. Four colony and cellular phenotypes of *C. albicans* NRRL Y-27077. Colony and cellular morphology of the White (A, F), Light Pink (B, G), Dark Pink (D, H) and Rough Pink (E, I) phenotypes on yeast-extract peptone dextrose plates stained with 5 μm/mL phloxine B after 5-10 days at 25 °C.](image-url)
In order to assess the influence of glucose concentration, temperature and incubation period on the ability of this strain to produce different colony phenotypes a 2X2X3 factorial design was conducted as indicated in Fig. 2. It should be noted that this data was derived from undifferentiated cell types. The percentage of the different cell types depicted in Fig. 2 was therefore derived from cultures taken directly from the glycerol stocks or the yeast culture collection without differentiation, suggesting that the initial phenotype was not yet determined. After incubation at 25 °C, the two dominant colony phenotypes were white and LP. After 3 days the colonies on 0.4 and 2% Glc YPD were mostly white (> 81%), with a low percentage LP colonies. However, when the glucose concentration was increased to 4%, no white colonies were observed after 3 days and most colonies were LP (circa 80%).

Upon further incubation (total time of 5 days) at the two lower glucose concentrations i.e. 0.4 and 2% Glc YPD, the number of white colonies decreased significantly, with a concomitant increase in LP colonies. The increase in incubation time also resulted in the appearance of a few DP and RP colonies. Increasing the incubation time in the presence of a higher (4%) glucose concentration also resulted in a change in the observed colony phenotypes. The LP and DP colonies disappeared and almost all the colonies were white after 5 days. At 37 °C most of the colonies were either white or LP at low glucose concentration. The most notable effect of increasing the incubation temperature was the observation of RP colonies, especially at glucose concentrations of 2% and higher. These colonies also increased with an increase in incubation time. Another intriguing observation was the absence of the DP colony phenotype on all glucose concentrations at 37 °C, indicating that it may only be formed under specific conditions.

**Fig. 2.** Percentage of colonies formed from mixed cultures at 25 °C and 37 °C after 3 and 5 days. Values are the mean of three independent experiments.
4.2 White-gray-opaque-like cells differ from previously described cell types

Several experiments were performed in order to determine whether the observed colony phenotypes correspond to any of the previously reported phenotypes. Our results indicated that the light, dark and rough pink colony phenotypes both resemble and differ from the gray, GUT and opaque cell types, previously described by other groups (Pande et al., 2013; Tao et al., 2014a) in a number of phenotypic aspects (Table 1). Although the smooth colonies appear light and dark pink on phloxine B plates, similar to the gray and opaque colonies respectively, the light pink colonies appear white as opposed to gray on YPD without phloxine B while the dark pink colonies usually do not appear flat on YPD agar medium (Miller & Johnson, 2002; Tao et al., 2014). The ultrastructural morphology of the different cell types also differ from those of the gray and opaque cells. Although the light, dark and rough pink cells appeared elongated, none of the cells produced surface pimples (Fig. 3A). Cells from RP colonies produced extracellular material instead, which may cause the rough appearance of the colonies. Intriguingly, white cells appeared elongated as opposed to the round and oval typical morphology of traditional white cells. We argue that this might be due to the fact that these cells were in the process of switching to other phenotypes. Furthermore, after 5 days on synthetic/defined media containing YNB and 1.25% glucose, RP colonies were wrinkled, although no hyphal cells were observed, while LP colonies were wrinkled with pseudohyphae (Fig. 3B). This data suggest that the white-gray-opaque-like system may also possess a unique filamentation programme different from the white-opaque system. In addition, in contrast to the differential expression of SAPs by gray and opaque cells on BSA agar (Tao et al., 2014a), none of the cell types reported here displayed any halos on BSA agar, indicating little or no SAP activity (results not shown).

The GUT cells exhibit a transcriptional profile that appears to be solely triggered in the digestive tract due to Wor1p overexpression only observed in vivo (Pande et al., 2013). This suggests that the GUT cells require specialized culture conditions to be induced in vitro (Pande et al., 2013). Phenotypes reported in this study were found to exist in vitro, although the DP phenotype became more pronounced in the presence of 5% CO₂ at 37 °C (see section 4.3), conditions which closely resemble those found in the gastrointestinal tract (Huang et al., 2009; Huang, 2012). These cells are also bigger and elongated (Fig. 4A), similar to the GUT cells. In contrast to the GUT cell colonies, colonies of the DP cells did not appear dark brown on YPD agar plates without phloxine B and they were stable for several days in vitro.
<table>
<thead>
<tr>
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<th>White</th>
<th>Gray</th>
<th>Opaque</th>
<th>GUT</th>
<th>Light pink</th>
<th>Dark pink</th>
<th>Rough pink</th>
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<sup>1</sup>This study, <sup>2</sup>Tao et al., 2014a, <sup>3</sup>Pande et al., 2013, <sup>4</sup>Si et al., 2013, <sup>5</sup>Ells et al., 2011, <sup>6</sup>Nobile & Mitchell, 2005; N/A – not applicable.
Fig. 3. Features separating the white-gray-opaque-like from white-gray-opaque cell types. A. SEM images of the white (Wh), light (LP), dark (DP) and rough pink (RP) cells after 5 days on YPD medium at 25 °C. B. Colony morphology of the rough pink and light pink phenotype and short connected hyphae of the light pink colonies on 1.25% Glc YNB after 5 days at 25 °C.

4.3 Transition from cell types of the white-gray-opaque-like system
Candida albicans NRRL Y-27077 displayed different colony phenotypes on YPD medium supplemented with varying concentrations of glucose. These were mainly the smooth (white, light and dark pink) and rough phenotype. The question that arose was whether these colony phenotypes can switch to other phenotypes under previously defined conditions used for white-gray-opaque switching (Tao et al., 2014) or under unique conditions. To answer these questions, different colony phenotypes were subjected to different environmental cues in order to assess the influence these cues have on white-gray-opaque-like morphological transition.

4.3.1 The role of temperature and glucose concentration
Low temperature is known to induce white-gray-opaque switching in C. albicans under the regulation of key transcription factors, Efg1p and Wor1p (Tao et al., 2014). The switching frequencies of the white-gray-opaque-like system in air at 25 °C and 37 °C are depicted in Figure 4A. Similarly to the white-gray-opaque switching reported by Tao and co-workers (2014a), we observed switching at both these
temperatures. The LP phenotype was the most stable, and only switched to another phenotype at low glucose concentration at 25 °C or high glucose concentration at 37 °C. This indicates the complex interaction between glucose concentration and temperature in this switching system. It was also observed that switching between RP and DP was a rare occurrence, only observed at 25 °C on 2% glucose YPD. It will be of interest to see what the effect of glucose concentration is on other switching systems reported for other C. albicans strains.

4.3.2 The influence of N-acetylglucosamine
Glucose and N-acetylglucosamine (GlcNAc) are known to induce white-gray-opaque switching at 25 °C in chemically defined (synthetic) medium in air (Huang et al., 2010; Tao et al., 2014a). Switching frequencies obtained from GlcNAc YNB agar are depicted in Figure 4B. This media partially destabilized the LP phase resulting in circa 50% of the colonies switching to the white phase, which became stable under these conditions. Substitution of GlcNAc for Glc in this synthetic medium resulted in a similar picture, with all phenotypes switching en masse to the white phase, however the white phenotype was not stable, switching back to LP. These results imply that although carbon source plays an important role in switching frequencies, the colony phenotypes are also influenced by nitrogen sources. This should be further investigated using Lee’s medium.

4.3.3 The role of 5% CO₂
A combination of 5% CO₂ and media that promote morphological switching has previously been shown to greatly regulate white-opaque and white-gray-opaque switching in C. albicans (Huang et al., 2009; Tao et al., 2014a). Switching frequencies in the presence of 5% CO₂ at 37 °C are depicted in Figure 4C. Similar to switching in air, the LP phenotype was stable in YPD, however this was independent of glucose concentration. The presence of CO₂ did not stabilize any other phenotype on YPD.

4.3.4 The role of host-like conditions
N-acetylglucosamine and CO₂, which are both available in the host, are known to influence white-gray-opaque switching in C. albicans (Tao et al., 2014a). Similar to switching in air, growth on synthetic media (1.25% Glc YNB) in CO₂ destabilized the LP phenotype, causing a switch to DP colonies (Fig. 4C). However, when GlcNAc was substituted for Glc, the LP colonies switched to white. Interestingly, the combination of 5% CO₂ and GlcNAc, resulted in the most complicated switching behaviour observed for all the tested conditions. Two overlapping switching systems can be seen: 1) DP ↔ white ↔ LP / DP → LP; 2) RP → white ↔ LP / RP → LP. This suggests that the white phenotype is an intermediate phase between the DP and LP phenotype as well as between the RP and LP phenotypes.
Switching in air at 25 °C

Switching in air at 37 °C

Switching in 5% CO₂ at 37 °C

Fig. 4. Phenotypic switching frequencies in the white-gray-opaque-like system. Switching frequencies after 5 days at 25 °C and 37 °C (A-B) and at 37 °C in the presence of 5% CO₂ (C).

4.4 The effect of white-gray-opaque-like switching on virulence factors

4.4.1 Effect on germ tube and biofilm production

Although is not clear whether gray cells filament under the same conditions as white cells, opaque cells require unique conditions which include growth on sorbitol and minimal medium with or without amino acids or with a low phosphate concentration as well as 1.25% GlcNAc at 25 °C (Si et al., 2013). In contrast to the opaque phenotype, the LP, DP and RP cells produced pseudo hyphae instead of true germ tubes in response to serum and Spider medium at 37 °C (Fig. 5A), conditions known to promote filamentation in white cells. There were, however, quantitative differences in the ability of the different cell types to form germ tubes in YPD supplemented with serum at 37 °C (Fig. 5B). Most notably, only white and RP cells formed hyphae at 25 °C. Moreover, the DP phenotype formed significantly less germ tubes compared to other cell types at 37 °C, suggesting that it may require unique culture conditions to optimize filamentation. In contrast, the RP cells produced most hyphae at both temperatures. We then asked whether these different cell types can form biofilms under the same conditions as white cells. According to our data, all the white-gray-opaque-like cell types are capable of forming biofilms at 25 °C and 37 °C after 48 h in RPMI-1640 medium (Figure 5C). Changes in temperature did not influence the ability of the white cells to form biofilms, but did have an influence on the biofilm forming abilities of the other cell types. This suggests that some cell types may be better suited to biofilm formation in different host niches.
4.4.2 Effect on fluconazole resistance

In addition, we also assessed resistance of biofilms from different cell types to the most clinically prescribed azole drug, fluconazole (FLC), in order to understand their role in drug resistance. Interestingly, the different cell types were inhibited by FLC at different levels, with the DP cells being most inhibited (*circa* 50% inhibition; \( p = 0.001 \)) at 25 °C. The white and LP cells were less inhibited (*circa* 25% and 33%, respectively), although this trend was not statistically significant \(( p \geq 0.05)\). The RP cells were resistant to FLC, as no statistically significant inhibition was observed \(( p = 0.2)\). At 37 °C, higher percentages of inhibition were obtained for all the cell types. Biofilms of the DP cells were inhibited by *circa* 73%. The change in temperature resulted in inhibition of biofilms formed by the white (*circa* 86%) and LP cells (*circa* 87%). The biofilms of the RP cells were again the most resistant with no statistically significant inhibition observed \(( p \geq 0.05)\). It is conceivable that the remainder of these cells may lead to formation of resistant biofilms or re-establishment of infections in the host after treatment.
**Fig. 6.** The effect of the white-gray-opaque-like system on fluconazole resistance. Growth after 48 h in RPMI-1640 at 25 °C (A) and 37 °C (B).

### 4.4.3 Effect on PGE₂ production

*Candida albicans* can produce prostaglandin E₂ (PGE₂) from arachidonic acid (AA), a polyunsaturated fatty acid derived from linoleic acid (Castro et al., 1994). It is classified as a virulence factor, because it is capable of inducing germ tube production by increasing levels of cAMP (Kalo-Kelin & Witkin, 1990; Noverr et al., 2001; Noverr & Huffnagle, 2004; Ells et al., 2011). However, biosynthesis of PGE₂ and its regulatory role in morphogenesis is not well understood. So far, a fatty acid desaturase, Ole2p, and the multicopper oxidase, Fet3p, are known to play important roles in PGE₂ production in *C. albicans* (Erb-Downward & Noverr, 2007). However, knockout mutant strains of these genes produce low PGE₂ levels, pinpointing to the involvement of other proteins and the complexity of PGE₂ production pathway in this yeast. Although it is known that *C. albicans* biofilms produce significantly more PGE₂ than planktonic yeast cells (Alem & Douglas, 2005), there are currently no reports on PGE₂ production from other cell types such as opaque cells. *Candida albicans* NRRL Y-27077 is capable of generating four different colony phenotypes, all with the ability of forming biofilms after two days, permitting reliable PGE₂ determinations and comparative analysis of this metabolite between these cell types. Previously, the same strain was used to measure PGE₂ production by enzyme-linked immunosorbent assay (Ells et al., 2011). However, in the culture used by these authors no cell type differentiation was conducted. Our study measured PGE₂ production by biofilms formed by the four separated cell types of this strain using LCMS/MS. Although our results presents lower levels of PGE₂ (Fig. 7), compared to previously reported data, some interesting observations were made. Consistent with
the fact that the different cell types formed biofilms at 25 °C and 37 °C, they also produced PGE$_2$ at both these incubation temperatures. Incubation temperature played a role in the amount of PGE$_2$ produced by the different cell types. Biofilms produced by the white cells, produced less PGE$_2$ at 37 °C than at 25 °C, however, biofilms produced by the RP cells, showed a significant increase in PGE$_2$ production with an increase in incubation temperature (Fig. 7). This may again point to the ability of different cell types to produce different levels of virulence factors in different host niches.

![Graph showing PGE$_2$ production at 25 °C and 37 °C](image)

**Fig. 7.** PGE$_2$ producing ability of the white-gray-opaque-like cell types. PGE$_2$ production was measure at 25 °C and 37 °C in RPMI-1640 medium and calculated on the basis of cell biomass.

5. **ORF19.4612 plays a crucial role in the white-gray-opaque-like switching system**

5.1 **ORF19.4612 plays a significant role in formation of different colony phenotypes**

Before we could examine switching between the wild type and isolates of ∆orf19.4612, we determined whether deleting ORF19.4612 had an influence on growth in YPD medium. According to this data, the mutation did not influence growth since all mutant strains displayed growth similar to the wild strain (Fig. S2). We then assessed the presence of different colony phenotypes in ∆orf19.4612 under various switching culture conditions (Tao et al., 2014a). Data of the prevalence of these different phenotypes at 25 °C and 37 °C after 5 days are depicted in Fig. 8. From this figure, it can be seen that deleting one copy of ORF19.4612 influenced the relative amounts of all the colony phenotypes at 25 °C, with the biggest difference seen for the white and DP colony types on 4% Glc. At 37 °C, no significant difference was observed in the ability to form white colonies or DP colonies. At this temperature deleting one copy of ORF19.4612 influenced the formation of LP and RP colonies. This indicates that ∆orf19.4612 strains are capable of forming all the different colony phenotypes observed for the wild type. In addition, deleting one copy of this ORF has an observable influence on the relative amounts of the different phenotypes observed under different conditions.
Fig. 8. Heat map depicting colonies formed from mixed cultures after 5 days at 25 °C and 37 °C. Strains were cultured on yeast-extract peptone supplemented with 0.4%, 2% and 4% glucose.

5.2 ORF19.4612 plays a significant role in switching between different cell types

5.2.1 ORF19.4612 influences switching between light pink and dark pink colonies at physiological temperature

Since ORF19.4612 was found to be significantly up-regulated during AA treatment at 37 °C (Ells, 2011), we analyzed switching in the mutant of this gene at the same physiological temperature (Fig. 9). When comparing the switching between the wild type strain and ∆orf19.4612 in air, the most striking difference observed is in the switching behaviour of LP to DP colonies (Fig. 9). In the wild type strain, the LP phenotype is stable at low Glc concentration and only circa 62% of the colonies switch to the DP phenotype at 4% Glc. This stability of the LP phenotype in ∆orf19.4612 is maintained at 0.2% Glc, with only circa 1% of LP colonies switching to DP. However, as the Glc concentration increases, the LP phenotype becomes more unstable. Interestingly, in the presence of 5% CO₂, high Glc concentration also causes a destabilisation of the LP phenotype with all the colonies switching to the DP phenotype in ∆orf19.4612 (Fig. 10).
Fig. 9. Switching between the wild type and Δorf19.4612 in response to physiological temperature, 37 °C, after 5 days on yeast-extract peptone dextrose agar supplemented with various glucose concentrations.

Fig. 10. Switching in response to 5% CO₂ at the physiological temperature after 5 days on yeast-extract peptone dextrose agar supplemented with various glucose concentrations.

5.2.2 Switching between light and dark pink in Δorf19.4612 is also affected by host-like conditions

Many authors have used mammalian hosts to analyze white-opaque switching and its effect on colonization (Pande et al., 2013; Xie et al., 2013; Tao et al., 2014a). Some studies have found that carbon sources such as GlcNAc and the presence of CO₂ influence switching (Huang et al., 2010; Xie et al., 2013). Therefore, we determined the role of ORF19.4612 in switching in the presence of host-like conditions using Glc, GlcNAc, 5% CO₂ and 37 °C (Fig. 11). According to this data, when glucose is used as a carbon source, the biggest influence of deleting one copy of ORF19.4612 was again observed in the switching between LP and DP. Under these conditions, the LP phenotype was more stable compared to the wild type. This
is in contrast to the observations on YPD agar, further highlighting the possible role of nitrogen sources or other media components in this switching system. When GlcNAC was used as a carbon source, a complex switching system can be observed in the wild type strain. However, deletion of one copy of ORF19.4612 perturbs this complexity and a simple switching system that also leads to LP colony formation can be observed. Taken together with the data on Glc YPD, there is an indication that ORF19.4612 may play a specific role in switching between LP and DP phenotypes. The mechanisms behind this, as well as the clinical significance will have to be further investigated.

Fig. 11. Switching in response to host-like conditions after 5 days at 37 °C.

5.2.3 ORF19.4612 plays an important role in filamentation in white-gray-opaque-like switching
The wild type strain and Δorf19.4612 strains also display differences in filamentation on various hypha-induction media. Consistent with increased border filamentation and agar invasiveness of Δorf19.4612 on certain nitrogen sources such as (NH₄)₂SO₄, L-alanine, L-leucine, L-isoleucine, L-methionine, L-ornithine and L-proline at 25 °C, 30 °C and 37 °C (Fig. 12A), Δorf19.4612 colonies were more filamentous than the wild type colonies on YPD agar plates after 5-7 days (Fig. 12B). However, when we subjected these strains as undifferentiated cultures i.e. cultures containing mixed phenotypes, to conditions that promote a quick response to hyphal-inducing cues i.e. incubation for a short period of time using liquid media that promotes hyphal formation, Δorf19.4612 differed from the wild type strain. For instance, these strains were more filamentous on liquid Lee’s medium supplemented with Glc and Spider medium while the wild type strain was more filamentous on YPD serum and Lee’s
medium supplemented with GlcNAc (Fig. 12C). We argued that the activity of **ORF19.4612** in hyphal formation is affected by different culture conditions as was the case in white-gray-opaque switching. Interestingly, when the differentiated cell types were analyzed for hyphal production in the presence of YPD serum, white, LP and DP cells of ∆orf19.4612 were more filamentous than those of the wild type strain, with only the RP cells being less filamentous (Fig. 12C). Consistent with this, ∆orf19.4612 colonies were highly wrinkled in the presence of YNB Glc and its colonies were filamentous on 0.4, 2 and 4% Glc YPD after 7 days (Fig. 12D). The wild type strain lacked these properties under these culture conditions. These results suggest that **ORF19.4612** plays an important role in filamentous growth during short term i.e. after 4 h in liquid media, and long term responses i.e. after 7 days on solid medium.

**Fig. 12.** The role of **ORF19.4612** in filamentation. A. Growth of mixed cultures at 30 °C after 7 days on minimal medium supplemented with amino acids as sole nitrogen sources. Broken edges represent nitrogen sources where ∆orf19.4612 display more invasiveness than the wild type strain. B. Filamentous growth in mixed cultures after 5 and 7 days on yeast-extract peptone dextrose agar. C. Quantitative analysis of hyphal formation after 4 h. D. Filamentous growth on various switching media.
5.2.4 ORF19.4612 plays an important role in PGE₂ production

Since ORF19.4612 was significantly up-regulated during PGE₂ production prior treatment with AA (Ells, 2011), we analyzed the role of this gene in this process as indicated in Fig. 13. Our data indicates that ORF19.4612 plays a role in enhancing PGE₂ production in RP cells at 37 °C. The RP cells of Δorf19.4612 produced significantly less PGE₂ at 37 °C than at 25 °C while the opposite was true for the wild type strain. This becomes significant when seen in the light of the fact that after 3 days on 0.4% Glc YPD at 37 °C, the RP colonies of the wild type are relatively stable (only circa 25% switch to other phenotypes), while deleting one copy of ORF19.4612 causes almost all the RP colonies to switch to other phenotypes. Since RPMI-1640 medium also contains low Glc concentration and the cells in the study of Ells (2011) were monitored for up to 48h, we reason that this medium may also stabilize RP cells at 37 °C for the time period used, thereby leading to high levels of PGE₂ production under these conditions.

![Diagram](image)

**Fig. 13.** A. Switching after 3 days on 0.4% glucose yeast-extract peptone dextrose at 37 °C. B. Prostaglandin E₂ production from biofilms of rough pink cells of the white-gray-opaque-like switching system in RPMI-1640 at 25 °C and 37 °C after 48 h.

6. Conclusions

Phenotypic elasticity is an important trait for survival and pathogenesis in the yeast, *C. albicans*. Therefore, understanding which signals induce or promote currently known phenotypes is a stepping stone in discovering novel ones. This study has led to the discovery of additional phenotypes, namely, the light, dark and rough pink phenotypes, resulting in a novel switching system called the “white-gray-opaque-like system”. These phenotypes differ from the opaque, GUT and grey phenotypes in a number of aspects. But most strikingly, these cell types can produce hyphae and biofilms under conditions promoting white cell filamentation and biofilm formation. Importantly, biofilms from these different cell types differ in their ability to form PGE₂ and to respond to fluconazole. We also revealed the important role of a previously uncharacterized gene, ORF19.4612, in the yeast-hyphal transition and white-gray-opaque-like switching as well as PGE₂ production. So far, the fungal biosynthetic pathway of PGE₂ has not been fully elucidated. The observation that this metabolite is produced from biofilms of different cells types implies that its biosynthetic pathway or the regulation thereof might be more elaborate than we previously thought.
7. Future prospects

The bistable white-opaque switching was found to be regulated by the mating type locus in *C. albicans* (Hull & Johnson, 1999; Miller & Johnson, 2002) whereas the tristable white-gray-opaque switching was found not to be fully regulated by this locus (Tao et al., 2014a). Similarly to white and gray cells, GUT and mucin-exposed cell types, both of which superficially resemble opaque cells, seem to form independently of the *MTL* locus (Pande et al., 2013; Kavanaugh et al., 2014). Consequently, these cell types fail to respond to pheromone stimulus and are mating incompetent. In addition, white, opaque, gray, GUT and mucin-exposed follow a unique transcription profile. To fully understand what cell types of the WGOL system truly represent, we will conduct transcriptional profiling and/or RT-PCR analyses using RNA sequencing between white, opaque and white, LP, DP and RP cells of *C. albicans* NRRL Y-27077. Data generated here will provide insight into whether WGOL could be regulated by master regulators, Wor1p and Efg1p, which regulate white-opaque switching. In addition, we will further dissect the role of *ORF19.4612* in yeast-hyphal transition, WGOL switching and PGE2 production in the double deletion mutant strain of this gene. Tackling the molecular aspect of this switching system will provide invaluable insight in deeply understanding the extent to which it is involved in virulence.

WGOL switching will also be optimised on standard Lee’s medium. Currently, this medium supported slower growth of NRRL Y-27077. Filamentation patterns will be compared to patterns from well characterized clinical isolates using time course experiments and hyphal induction cues such as serum and GlcNAc. Furthermore, the *MTLa* and *MTLα* strains of NRRL Y-27077 will be treated with pheromones to determine formation of conjugation tubes usually formed during mating (Lin et al., 2013; Tao et al., 2014b). If cell types are mating competent, mating efficiencies will be determined. The biofilm-forming ability of WGOL switch phenotypes will be validated further by measuring biofilm thickness. All of these processes will be directly compared with the well characterized *C. albicans* strain, WO-1, in order to further validate the novel nature of WGOL system. The rationale behind using *C. albicans* strain WO-1 for comparison is that in the work of Slutsky and co-workers (1987), this strain was reported to display other colony phenotypes apart from white and opaque. Furthermore, we will isolate more clinical strains and analyze them for the presence of WGOL switching system since it is currently observed in one clinical isolate, NRRL-27077.

8. References


Supporting Information

**Fig. S1.** *C. albicans* NRRL Y-27077 on yeast-extract peptone dextrose agar after 5 days at 25 °C. Black and red arrows indicate opaque- and white-like colonies respectively.

**Fig. S2.** Growth analysis between the wild type strain and isolates of Δorf19.4612. Strains were cultured at 30 °C in yeast-extract peptone dextrose broth and growth was monitored every 90 min for 16 h.
Chapter VI – General Discussions/Conclusions

1. A general outlook on virulence factors
The human body is populated by members of the eukaryotic, archaea and bacterial domains (Pérez et al., 2013; Pérez & Johnson, 2013; Fox et al., 2014). *Candida albicans*, the most frequently isolated eukaryotic fungal species from critically ill patients (Zaborin et al., 2014), is well studied due to its ability to cause devastating diseases. Although the focus has previously been placed on understanding disease causing mechanisms, recently the commensal, non-pathogenic behaviour of this yeast has also become the subject of research (Cheng et al., 2011; Pande et al., 2013; Pérez et al., 2013; Pérez & Johnson, 2013; Kavanaugh et al., 2014). As a result, an important light on how *C. albicans* alternates between these two states has been cast. On the one hand it appears that the host provides key signals that suppress virulence (Pande et al., 2013; Kavanaugh et al., 2014) while on the other, it provides signals that elicit the pathogenic state (Nobel et al., 2010; Kavanaugh et al., 2014). However, it is clear that critical illness sets a platform for pathogenesis and infection (Zaborin et al., 2014). More often than not, the alternation between the commensal and disease-causing traits of *C. albicans* is characterized by a switch from the yeast to the hyphal state or the epigenetic switch from the typical white to the mating competent opaque phase (Hull & Johnson, 1999; Miller & Johnson, 2002; Huang, 2012; Ene & Bennett, 2014; Soll, 2014; Tao et al., 2014a). Both these switches are important for the inherent capacity of *C. albicans* to cause clinical and subclinical infections. While the yeast cells are easily scattered and delivered into blood where they can cause bloodstream infections, the hyphal cells are more invasive and better at tissue penetration (Sudbery & Berman, 2004; Sudbery et al., 2011). Both the yeast (white and opaque) and hyphal cells contribute, via specific signaling pathways, to biofilm formation which accounts for a large number of device-associated clinical infections (Nobile & Mitchell, 2005, 2006; Fox & Nobile, 2012; Nobile et al., 2012; Lin et al., 2013; Fox et al., 2014; Soll, 2014; Nobile & Johnson, 2015). Recent reports suggest that white a cells facilitate opposite- and same-sex mating in opaque cells by exchanging mating factor (Mf) a and α pheromones and this seems to further enhance genetic diversity and confer novel traits with increased antifungal resistance owing to extent of aneuploidies (Ene & Bennett, 2014; Tao et al., 2014b). Opaque colonies themselves are better colonizers of the skin and they can cause skin cavitations due to opaque-specific synthesis and secretion of hydrolytic enzymes (Kvaal et al., 1997, 1999; Miller and Johnson, 2002; Soll, 2014). However, given the chance to colonize the host internal organs, opaque cells can become invisible to immune attack and phagocytosis due to their inability to release a potent chemoattractant (Geiger et al., 2004; Sasse et al., 2013). As a result, they can be disseminated to parts of the host where they can switch back to the more pathogenic white state (Lohse & Johnson, 2009; Lohse et al., 2013; Ene & Bennett, 2014).

2. Arachidonic acid and PGE₂ are emerging virulence factors
Another important virulence factor is the ability of *C. albicans* to liberate and utilize arachidonic acid (AA), an omega-3 polyunsaturated fatty acid, from the host and to produce immunomodulatory molecules such as prostaglandin E₂ (PGE₂) (Noverr et
In the host, *C. albicans* can release AA from damaged tissues by utilizing a panel of lipolytic enzymes which are also considered important virulence factors. Given that AA is found almost in any cell type in the host, AA serves as an important carbon source that is a readily available precursor for PGE$_2$ production. Due to immunomodulatory capabilities of PGE$_2$, miss-regulation of the immune system can cause unwarranted inflammatory responses which may benefit *C. albicans* to cause infections. In addition, *C. albicans* can sense PGE$_2$ from its surroundings and a transcriptional response can be triggered which can remodel the phenotypic response towards the specific host niches (Levitin and Whiteway, 2007). Therefore, AA and PGE$_2$ are considered important virulence factors in *C. albicans*.

3. **Arachidonic acid triggers a unique stress response system**

Although AA does not form part of the lipid component of *C. albicans*, it is capable of triggering a unique transcriptional response in this yeast (Ells, 2011). To better understand this AA-induced transcriptional response, we studied genes that were significantly up-regulated by analyzing their deletion mutants. Among those we analyzed were the *HST6*, *SSY1* and *ORF19.4612* genes. The *HST6* gene is a functional homologue of *S. cerevisiae* *STE6*, which encodes a plasma membrane-bound protein required for the secretion of the mating factor α pheromone (Raymond et al., 1998; Magee et al., 2000, 2002). As a pheromone export gene, *HST6* is up-regulated, along with other sex-specific genes, during pheromone response and mating (e.g. Tao et al., 2014b). However, the fact that Hst6p was found to be expressed in all cell types in *C. albicans* (Raymond et al., 1998) suggests that it participates in biological processes in a general sense. Consistent with this prediction, our phenotypic data strongly suggest that Hst6p plays an important role in stress protection against a wide range of stress-inducing compounds. This data fits well with the context of exposing *C. albicans* to AA, known to cause predisposition to environmental stress by causing an increase in antifungal susceptibility, by altering the saturation level and fluidity as well as the ergosterol content of cell membranes (Ells et al., 2009). In addition, this exposure causes sensitivity towards oxidative stress which leads to programmed cell death (Thibane et al., 2012). Therefore we maintain that up-regulation of *HST6* during exposure to AA was part of a stress response system employed by *C. albicans*. Interestingly, the work of Ells (2011) also revealed the significant up-regulation of the *SNQ2* gene, also an ABC transport protein encoding. In *S. cerevisiae*, Snq2p plays a protective role against metal ion stress (e.g. Na$^+$, Li$^+$ and Mn$^{2+}$) as well as oxidative stress-inducing chemicals such as 4-nitroquinoline-N-oxide and methyl-nitro-soguanidine (Haase et al., 1992; Decottignies et al., 1995; Decottignies & Goffeau, 1997; Ververidis et al., 2001). Consistent with these findings are the roles of ABC proteins in counteracting oxidative damage in distal organisms such as *Caenorhabditis elegans* and *Salmonella enterica* (Liesa et al., 2012; Bogomolnaya et al., 2013). Exploiting and understanding the diverse roles of ABC transport proteins is of great importance in human genetics since they are tightly linked to important human genetic diseases. For instance, in Alzheimer’s or Parkinson’s diseases, which result as a cause of brain inflammation, expression of ABC transport proteins is highly regulated by oxidative stress (Hartz & Bauer, 2010). This suggests that the ABC stress response system is widely conserved across several domains of life.
4. Exposure to arachidonic acid exposes a link between stress and amino acid sensing

Our phenotypic data also implicates SSY1, from the SPS (Ssy1p, Prt3p and Ssy5) system, in stress response. The SSY1 gene, conserved in yeasts, is also a membrane-bound protein required for sensing of amino acids and other nitrogen sources. Although strongly implicated in yeast to hyphal transition and virulence (Brega et al., 2004; Davis et al., 2011; Vylkova & Lorenz, 2014), this gene has never been implicated in stress adaptation. We discovered that deleting SSY1 greatly affects colony and cellular morphology during exposure to conditions replete with ammonium and under nitrosative stress, promotes tolerance to osmotic, cell wall and heavy metal stress while resulting in sensitivity to heat stress and fluconazole treatment. This data suggests Ssy1p plays an important role in a differential stress response in C. albicans. Since SSY1 gene was significantly up-regulated during AA exposure in nutrient poor conditions, our phenotypic data also suggest that Ssy1p may have benefited C. albicans under these conditions. The microarray data (Ells, 2011) seems to suggest that SSY1 was expressed in response to pH changes which might have been orchestrated by conversion of AA into PGE₂. According to this data, after 8 h, both SSY1 and STP2 are significantly up-regulated with MEP2 being only up-regulated after 24 h. It is possible that Mep2p resulted in the utilization of ammonium incorporated in amino acids and the release of ammonia which can increase the pH in RPMI-1640 medium. Accordingly, after 48 h, RIM101, required in an alkaline pH, was significantly up-regulated which affirms the possible activity of SSY1, STP2 and MEP2 genes in increasing pH of the RPMI-1640 medium. A recent study has already determined that ΔΔstp2, disrupted for the downstream component of SSY1, is defective in pH regulation and yeast to hyphal transition in an acidic phagosomal environment due the inability to utilize amino acids and to liberate ammonia to alkalinize the acidic environment (Vylkova & Lorenz, 2014). Therefore, it is clear that early conversion of AA to PGE₂ may cause sudden shifts in the pH environment and C. albicans cells respond to these pH shifts.

However, our phenotypic data suggests that up-regulation of SSY1 may have also been crucial to heat stress. This is because the Ssy1 mutant was sensitive to heat stress at 37 °C and 42 °C. These findings are consistent with literature (Ells et al., 2009), which demonstrates that AA causes a predisposition to stress and affects membrane fluidity. Furthermore, AA conversion to PGE₂ was conducted at 37 °C of which might have been stressful to cells exposed to AA.

Our data also suggest that SSY1 can result in sensitivity to conditions rich in a nitrogen source. Analysis of the wild type strain and ΔΔssy1 on conditions replete with a nitrogen source such as 10-100 mM ammonium sulphate [(NH₄)₂SO₄] and other nitrogen sources shed an important light in this regard. According to this data, SSY1 deletion resulted in normal cell growth under nitrogen rich conditions. Therefore, this deletion protected C. albicans mutant strain (ΔΔssy1) from effects of excess nitrogen. Similar observations were made during growth on nitrosative stress-inducing chemicals such as S-nitrosoglutathione and sodium nitrite. But since culture conditions that were applied during AA metabolism (RPMI) are poor in nitrogen and closely mimic those found in the host niches such as the bloodstream, the same cannot always be true. Therefore, it appears that Ssy1p is crucial under nitrogen poor conditions where it can activate hyphal formation to extract nutrients from distant locations, under nutrient rich conditions where it may result in sensitivity
to stress and regulate morphogenesis or under conditions lacking nitrogen sources where it may be repressed and result in stress tolerance.

5. **Arachidonic acid metabolism is linked to a novel switching system that facilitates its conversion**

In mammals, AA is converted to PGE$_2$ and this metabolite can modulate aspects of the immune and inflammatory responses such as crosstalk between dendritic and natural killer cells (Hirazi, 2013). Interestingly, PGE$_2$ is produced by different cell types of the immune system (Kalisnki, 2012). The analysis of the recently reported switching system, the white-gray-opaque-like (WGOL) switching comprising of the white, light pink (LP), dark pink (DP) and rough pink (RP) cell types, reveals that PGE$_2$ can also be produced by specialized cell types in the pathogenic yeast *C. albicans*. Further revealed by this data is that PGE$_2$ production is not limited to white cells and host temperature. We have demonstrated that PGE$_2$ can also be produced at lower temperatures, suggesting that skin colonizers can efficiently utilize AA and produce PGE$_2$. This is of great importance since throughout this study we utilized a strain isolated from the skin (NRRL Y-27077). It will be interesting to analyze the ability of this strain to undergo white-gray-opaque-like switching using the *ex vivo* (skin) infection model.

Interestingly, only the RP cells produced significantly more PGE$_2$ at 37 °C than at 25 °C, suggesting that they may be better suited for PGE$_2$ production in the host. Moreover, the mutant of *ORF19.4612*, currently uncharacterized, was found to be defective in RP cell formation at 37 °C after 3 days and switched *en masse* to LP and DP cell types on 0.4% glucose (Glc) yeast-extract, peptone and dextrose (YPD) medium while 75% colonies of the wild type strain remained in the RP phenotype. Interestingly, *ORF19.4612* was significantly up-regulated after 8 h, 24 h and 48 h when *C. albicans* was treated with AA, which led us to suggest that it plays an important role in WGOL switching in the presence of this fatty acid. The fact that RPMI-1640 medium contains 0.2% Glc suggests that it may also stabilize RP cell formation to some degree like 0.4% Glc YPD, while 3 days is more than enough time for biofilms to mature and produce PGE$_2$. Therefore, phenotypic switching data seems to strongly suggest that up-regulation of *ORF19.4612* was necessary for RP cell stabilization leading to efficient conversion of AA to PGE$_2$.

6. **References**


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**Summary**

*Candida albicans* is a permanent resident of the healthy microbiome of humans. It co-inhabits the host micro-environment with trillions of species which make up the complete microbiota. Host parameters such as nutrient availability, CO$_2$, and temperature as well as pH fluctuations are some of the key contributory elements to *C. albicans* phenotypic attributes and virulence. In this study, our analysis was based on arachidonic acid (AA), a polyunsaturated fatty acid, as an inducer of a genome wide transcriptional response. Arachidonic acid is a precursor of prostaglandins (PGs) such as PGE$_2$ which can act on prostanooid receptors and potentially alter important biological functions. Since AA is available in the host, it is of great importance to understand the response of pathogenic yeasts to AA and how this interaction contributes to clinical infections.

We analyzed genes up-regulated during AA exposure of *C. albicans* biofilms. Three mutants, two disrupted for membrane-bound proteins (ABC pheromone exporter transport, Hst6p, and amino acid sensor, Ssy1p) and ORF19.4612 (uncharacterized) were analyzed using phenotypic microarrays (PMs). Additional roles of Hst6p were uncovered in this study, one of which is linked to its up-regulation in the presence of AA. The PM data suggests that Hst6p is required for stress protection and conventional phenotypic screens confirmed this. ∆∆hst6 was found to be sensitive to osmotic, oxidative, cell wall and heavy metal stress. Because AA can induce sensitivity to stress, our phenotypic data suggest that HST6 was up-regulated during AA exposure, to respond to stress. In addition to HST6, other ABC transport genes, also important in stress, were up-regulated. Other groups have recently discovered the role of these proteins in stress in higher organisms such as humans, suggesting that the ABC-stress response system is widely conserved. Another function of Hst6p was suppression of hyphal formation in conditions replete with nitrogen sources. ∆∆hst6 was filamentous under these conditions. In addition, ∆∆hst6 formed highly wrinkled colonies with filamentous borders, whose cells were filamentous, under nitrosative stress. Therefore, our data supports the fact that Hst6p is expressed in all the cell types in *C. albicans* and has a more generic role as opposed to pheromone export alone.

Analysis of phenotypes also revealed additional functions of the amino acid sensor, Ssy1p. According to literature, Ssy1p activates cleavage of Stp1p and Stp2p in response to lower pH and poor nutrient availability. In turn, these proteins activate amino acid permeases, Mep1p and Mep2p, required for pH neutralization and yeast-hyphal transition respectively. We found that under stressful conditions, Ssy1p results in differential stress response. For instance, while the wild type strain cells were inflated, cells of ∆ssy1 grew normally on high ammonium concentrations and in the presence of nitrosative stress. In addition, ∆∆ssy1 was resistant to osmotic and cell wall stress. Furthermore, ∆ssy1 was sensitive to heat stress at 37 °C and 42 °C. Since AA conversion to PGE$_2$ was conducted at 37 °C, and this fatty acid results in predisposition to stress which may affect membrane fluidity, we argue that SSY1 was up-regulated in response to heat stress.

Although the microarray data failed to reveal genes directly involved in PGE$_2$ production, the analysis of the white-gray-opaque-like (WGOL) switching, suggest that up-regulation of ORF19.4612 was necessary for rough pink (RP) cell formation and conversion of AA into PGE$_2$. At 37 °C in RPMI-1640 medium, RP cells of the wild type strain produce significantly more PGE$_2$ from AA. ∆orf19.4612 is defective in RP cell formation after 3 days on conditions similar to those of RPMI-1640 medium. Since ORF19.4612 was expressed after 48 h at 37 °C, is therefore not only implicated in WGOL, but also in PGE$_2$ production.

**Keywords:** Candida albicans · Nutrient sensing · Phenotypic switching · PGE$_2$ production · Pathogen · Stress response
**Opsomming**

*Candida albicans* is 'n permanente lid van die mikrobiom van gesonde mense. Dit deel die gasheer se mikro-omgewing met triljoene ander spesies wat saam deel uit maak van die totale microbiota. Toestande in die gasheer soos beskikbaarheid van voedingstowwe, CO₂, en temperatuur asook veranderings in pH is sommige van die sleutelelemente wat bydrae tot *C. albicans* se fenotipiese eienskappe en virulensie. In ons analyse in hierdie studie is gebaseer op arachidooonsuur (AS), 'n polionversadigde vetsuur, as induseerder van 'n genoomwyse transkripsionele respons. Arachidooonsuur is 'n voorloper van prostaglandiene (PGE₂) wat inwerk op prostanoïdeseptore en potensieel belangrike biologiese funksies kan beïnvloed. Aangesien AS in die gasheer beskikbaar is, is dit belangrik om die response van patogene giste op AS en hoe hierdie interaksie bydra tot kliniese infeksies te verstaan.

Ons het die gene wat opgereguleer is gedurende blootstelling van *C. albicans* biofilms aan AS, geanaliseer. Drie mutante, waarvan twee deles van membraangebonde proteïene (ABC feromooneksporpteleien, Hst6p, en aminosuursensor, Ssy1p) en ORF19.4612 (ongeëkarakteriseerde) is geanaliseer m.b.v. fenotipiese “microarrays”. Addisionele funksies vir Hst6p is in hierdie studie ontedek. een daarvan is gekoppel aan sy opregulering in die teenwoordigheid van AS. Die fenotipiese “microarray” data stel voor dat Hst6p nodig is vir beskering teen stress en konfensiolele stressstoetse beaam. δhst6 was sensitiw met onder hst6 onderhoud van omgaan met stikstofbronne. δhst6 was filament at hierdie toestande. δhst6 het ook geplooiëde kolonies met filamentagtige kante gevorm, waarvan di selle filamentagtig was, onder stikstofstress. Dus ondersteun ons data die feit dat Hst6p in alle selftipes van *C. albicans* uitgedruk word en 'n meer generiese rol speel as bloot feromoontransporter.

Analyse van fenotipies het di toon duidelik gemaak dat die aminosuursensor, Ssy1p, ook addisionele funksies het. Volgens literatuur, aktiveer Ssy1p die slyting van Stp1p en Stp2p in respons tot laer pH en voedingstofgebrek. Op hulle beurt aktiveer hierdie proteïele aminosuurpermeases, Mep1p en Mep2p, wat nodig is vir neutralisering van die pH en oorsakeling van gisagtige na filamentagtige groei, onderskeidelik. Ons het gevind dat Ssy1p lei tot transnisiële stressresponse onder stressvolle toestande. Terwyl die selle van die wilde tipe byvoorbeeld opgeblase was, het die selle van δsy1 normaal gegroei op hoë ammonium konsentrasies en in teenwoordigheid van stikstofstress. δsy1 was ook weerstandsbied van enthesele en selwandstress. δsy1 was egter sensitiw vir sittestress by 37 °C en 42 °C. Aangesien omskakeling van AS na PGE₂ teen 37 °C uitgeoer was en blootstelling aan hierdie vetsuur kan lei to stress wat membraanvloeibaarheid kan beïnvloed, voer on aan dat Ssy1 opgereguleer is in respons tot o.a. hittestress.

Alhoewel die transcriptie-analise nie gene wat direk betrokke was by PGE₂ produksie, kon aantoen nie, het die analyse van die wit-grys-ongeursig-tipe omskakeling, voorgestel dat opregulering van ORF19.4612 nodig was vir die vorming van growwe pienk selle en omskakeling van AS na PGE₂. By 37 °C in RPMI-1640 medium, vorm die growwe pienk selle van die wilde tipe beduidend meer PGE₂ vanaf AS. orf19.4612 vorm minder growwe pienk selle na 3 dae onder toestande soortgelyk aan die van RPMI-1640 medium. Aangesien ORF19.4612 uitgedruk was na 48 by 37 °C, word dit geimpliseer in wit-grys-ongeursig-tipe omskakeling en PGE₂ produksie.

**Sleutelwoorde:** Candida albicans · Voedingstofgewaarwording · Fenotipiese omskakeling · PGE₂ produksien · Patogeen · Stressresponse