

Pga1
A GPI-ANCHORED CELL WALL PROTEIN
NECESSARY FOR ADHESION AND BIOFILM
FORMATION *IN CANDIDA ALBICANS*

by

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**A thesis submitted in partial fulfillment
of the requirements for the degree of
Master of Science in Molecular Biology**

Under the supervision of
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ABSTRACT

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FORMATION IN *CANDIDA ALBICANS*

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The human fungal pathogen *Candida albicans* is one of the leading causative agents of death in immunocompromised individuals. Many factors have been implicated in virulence including filamentation inducing transcription factors, adhesins, lipases, and proteases. Many of these factors are GlycosylPhosphatidylinositol (GPI)-anchored cell surface antigenic determinant proteins. Pga1 is a short 133 amino acid protein shown to be up regulated during cell wall regeneration. The purpose of this study is to characterize the role of Pga1 as far as filamentation on solid and liquid filamentation and non-filamentation inducing media, susceptibility to cell surface disrupting agents, oxidative stress to a potentially lethal dose of hydrogen peroxide. Furthermore, virulence in a mouse model of disseminated candidiasis, adhesion to human epithelial cells and biofilm formation will be characterized. This will be performed by creating a homozygous *pga1* null strain and comparing the

phenotype to the parental strain. It was observed that the null phenotype was over filamentous on both liquid and solid potato dextrose agar (PDA) media compared to the parental strain at both 30°C and 37°C. In addition the mutant strain showed less oxidative stress tolerance. The mutant exhibited reduced susceptibility to sodium dodecyl sulfate (SDS), but increased susceptibility to calcofluor white, both being cell surface disrupting agents. However, no differences in response to Congo red or caspofungin were observed. Furthermore the mutant exhibited a 50% reduction in adhesion and a 33% reduction in biofilm formation compared to the parental strain, which was reflected as a reduction in virulence. This data is interesting; bearing in mind that disruption of many cell surface components usually weakens the cell wall, resulting in hypersensitivity to the utilized agents and a reduction in filamentation. Whether the cell compensates a *pga1* deletion and responds by up regulating other cell surface components is possible.

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Chapter 1

Introduction

Candida albicans, the major opportunistic fungal pathogen of humans (Figure 1), commensally colonizes virtually every anatomical site of the host from the oral cavity to the gastrointestinal and urogenital tracts, generating no obvious pathology.

However *C. albicans* can turn pathogenic. In fact, *Candida* species are the fourth leading cause of catheter-related bloodstream infections in hospitalized patients in intensive care units, and *C. albicans* is the species most commonly isolated from patients with these infections. *Candida* species are associated with severe and deadly systemic bloodstream infections with a high mortality rate (31.8%) in neutropenic patients (Chen et al., 2008) and with an attributive mortality rate approaching 35% in the United States. Approximately, \$1 billion per year is spent on antifungal treatments for patients with hospital-acquired *Candida* infections in the US (Kadosh et al., 2005; Banerjee et al., 2008).

Importantly, *C. albicans* can cause superficial as well as disseminated infections when changes in the host immune system or microflora arise. Clearly the immune status of the host strongly influences the ability of *C. albicans* to cause disease. In immunocompromised, individuals including those undergoing bone marrow and organ transplantation, cancer chemotherapy and those with primary or acquired immunodeficiency, *C. albicans* can cause life-threatening infections (Odds 1988; Tsao et al., 2009) that may arise as hematogenously disseminated infections or as localized primary diseases of deep organs (Plaine et al., 2008). *C. albicans* can establish bloodstream infections that can progress to deep-seated

infections (Figure 1) of major organs such as the kidney, liver and brain, many of which are fatal (Walker et al., 2009). Predisposing factors for *C. albicans* infections include immunosuppressive therapy, antibiotic therapy, human immunodeficiency virus infection, diabetes, and old age.

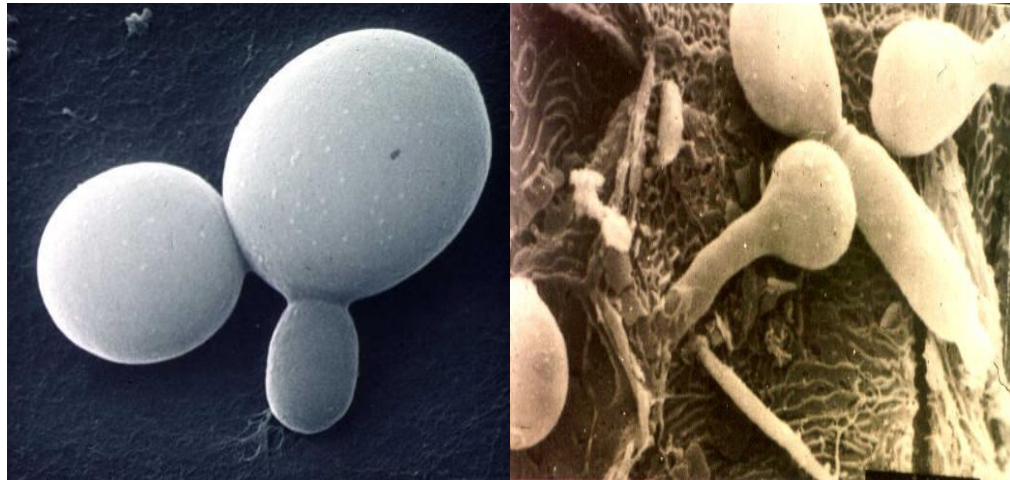


Figure 1. *Candida albicans* in different forms.

The non invasive yeast form is the major commensal form present in warm blooded animals. The filamentous form is necessary for tissue and organ invasion (www.overcomingcandida.com).

The number of clinical *C. albicans* infections worldwide has risen considerably in recent years, and the incidence of resistance to traditional antifungal therapies is also rising. Many existing antifungal therapies have clinical side effects; therefore new strategies are needed to identify new targets for antifungal therapy (Cao et al., 2009).

1.1 *Candida albicans* dimorphism

Candida albicans is a diploid asexual pleiomorphic fungus. It possesses several virulence properties including the ability to undergo a reversible morphological conversion from single ovoid budding yeast cells (blastospores) to elongated cells attached end-to-end (filaments) that are known to occur in two distinct forms: pseudohyphae and true hyphae (Figure 2). Pseudohyphal cells are elliptical in shape with constrictions at the septa, whereas hyphal cells have parallel sides showing a relatively uniform width and true septa lacking constrictions. Tissues infected with *C. albicans* typically contain a mixture of blastospores, pseudohyphae, and hyphae.

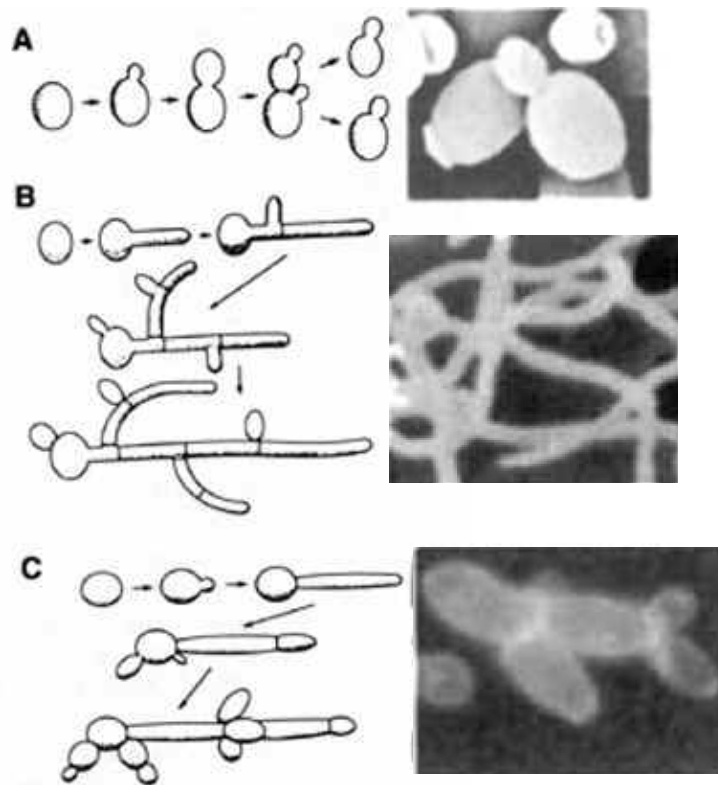


Figure 2. *Candida* from yeast to invasive forms

Dimorphic switching results in hyphal branching with an intermediate pseudohyphal form (www.overcomingcandida.com). A. Budding yeast, B. Hyphae, C. Pseudohyphae.

In addition, *C. albicans* has a special ability to form chlamydo spores, thick-walled cells, in certain environmental conditions such as nutrient-poor media, and low temperature (25°C to 30°C) where light and presence of glucose inhibit its formation. Chlamydo spores arise on elongated suspensor cells situated on pseudohyphae or hyphae (Figure 3). The physiological significance of chlamydo spores is not completely understood but they have been isolated from AIDS patients (Sonneborn et al., 1999).

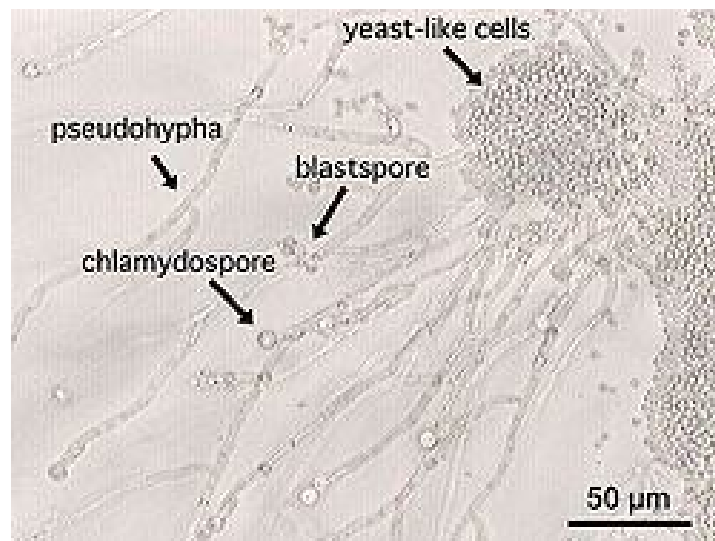


Figure 3. *Candida albicans* exhibiting chlamydo spore formation.

Microscopic image (200-fold magnification) of *Candida albicans* ATCC 10231, grown on cornmeal agar medium with 1% Tween80. (Tambe, 2005)

1.2 Dimorphism and virulence

C. albicans blastospore-to-filament transition is required for virulence. One of the features that allow for these transitions (Figure 4) from a commensal organism to a successful pathogen is its ability to sense complex environmental signals (Tsao et al., 2009). These signals relate to medium pH, nutrient type and availability, temperature, and concentration of atmospheric gases. In fact under a combination of conditions that include normal to slightly alkaline pH, host body temperature (37°C), physiological concentration of CO₂ (5%), hypoxia, presence of N-acetylglucosamine, serum, nutrient limitation and starvation especially glucose starvation, growth on certain synthetic cell culture media such as M199 pH7 or cornmeal agar, *C. albicans* has the ability to switch *en masse* from yeast to hyphae and eventually from a commensal to a pathogen, to induce cell proliferation signaling pathways and to induce the expression of virulence determinants (Calderone, 2002).

At 37°C and in serum, the most potent hyphal inducer, *C. albicans* initially form small projections termed germ tubes. Germ tubes are precursors of hyphal filaments and are actually used as a diagnostic tool in the identification of *C. albicans*. Subsequent cell division at the apical tip of the germ tube allows extended filament formation (Banerjee et al., 2008).

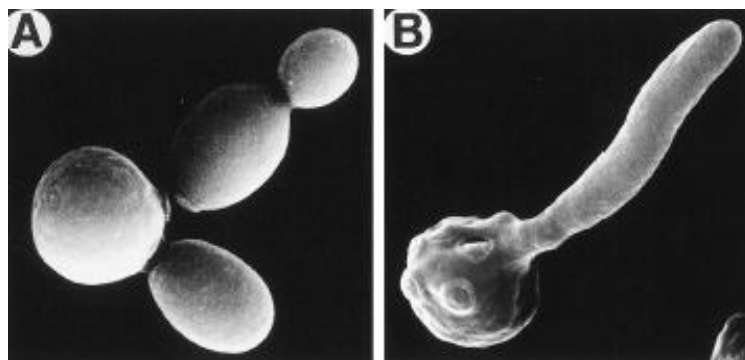


Figure 4. The basic bud-hypha transition in *C. albicans*.

A) Yeast form cells. B) Germ tube extension, a precursor to hyphal formation.

Note the bud scars on the mother cell. (Soll 2002)

This morphological switching is triggered through multiple signal transduction pathways that ultimately results in the activation of a complex transcriptional program resulting in filament formation. Filamentation has been long known to be coupled with virulence and pathogenesis in *C. albicans* as well as several other pathogenic fungi. A clear correlation between extensive invasion of oral epithelial mucosal surfaces and increased number of hyphal filaments is observed. Indeed, epithelial tissue invasion is believed to be caused by the mechanical force exerted by hyphal extension. *C. albicans* hyphal filament extension is critical for the process of thigmotropism that guides the hyphal growth along ridges of the substratum, which is believed to be an adaptation for tissue invasion, in addition to the ability of *C. albicans* to breach endothelial cells and lyse macrophages and neutrophils when endocytosed (Banerjee et al., 2008). The blastospore-filament transition is accompanied by the induction of many genes. While some of these genes are required for the morphological transition, others encode proteins that give the filamentous forms their distinctive properties. The fact that filamentation is necessary for virulence comes from many observations. First, deletion of key filamentation inducing genes such as *cph1* and *efg1* blocks filamentation under most growth conditions and result in an avirulent phenotype. Second, filamentous forms of *C. albicans* have been isolated from patients suffering from systemic candidosis. However this relationship between filamentation and virulence is not absolute. For example, the three major characterized negative regulators of filamentation under non-inducing conditions are Rfg1, Nrg1, and Tup1. In the well-studied baker's yeast, *Saccharomyces cerevisiae*, Nrg1 and Rox1-a protein similar to Rfg1 in structure, are sequence-specific DNA-binding proteins that recruit Tup1, a global transcriptional repressor to achieve gene silencing. Deletion of *rfg1*, *tup1*, or even *nrg1* result in lack of repression of filamentation genes and result in a constitutively filamentous strain under most media tested. Interestingly and

surprisingly, such strains are highly attenuated for virulence. Thus filamentation alone is not sufficient for virulence. It appears that the ability of *C. albicans* to switch between the blastospore and hyphal morphologies is necessary for virulence (Kadosh et al., 2005).

1.3 Biofilm formation

Transplantation procedures, the use of chronic indwelling devices, and prolonged intensive care unit stays have increased the prevalence of fungal disease. Substrates for biofilm growth associated with candidiasis include indwelling medical devices such as artificial joints, catheters, central nervous system shunts, dental implants, heart valves, ocular lenses, and vascular bypass grafts (Hawser and Douglas, 1994).

Biofilm formation proceeds in three distinct developmental phases: early (0 hr to 11 hr), intermediate (12 hr to 30 hr), and maturation (38 hr to 72 hr). Initially (0 to 2 hr), the majority of cells present are blastospores adhering to the surface. At 3 to 4 hr, distinct microcolonies start to appear and eventually form communities of thick tracks of fungal growth after 11 hr due to increased cell growth and aggregation along areas of surface irregularities. During the maturation phase, the amount of extracellular material (ECM) increases with incubation time, until *C. albicans* communities were completely encased within this material. Fungal communities and the extracellular material in which they are embedded constitute the biofilm (Figure 5). Filamentous hyphal cells are major constituents of biofilm. Antifungal resistance of biofilm-grown cells increases in conjunction with biofilm formation. This is observed when Minimum Inhibitory Concentrations (MICs) of certain anti-fungal drugs progressively increase as the biofilms develop and grow to maturation (Chandra et al., 2001).

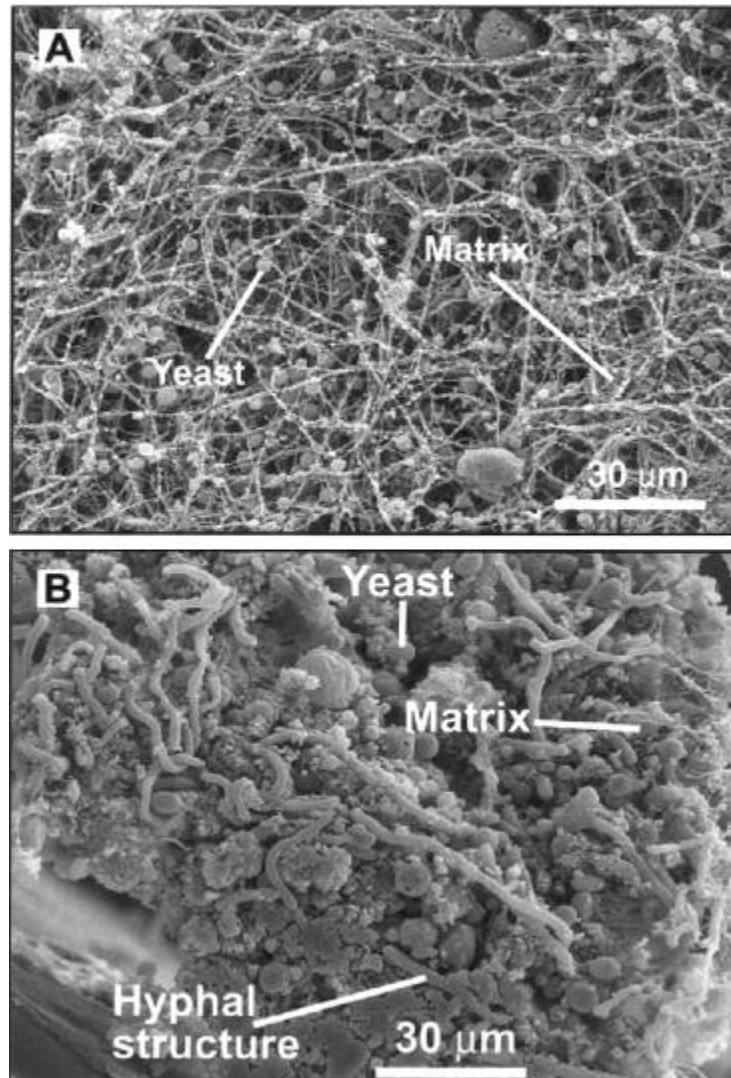


Figure 5. *Candida albicans* biofilm after 24 hours of development.

Catheter wall and intraluminal biofilm in an end-on orientation is shown. A) Yeast adherence to surface B) Biofilm formation after 24 hr. (Andes et al., 2004)

1.4 The fungal cell wall

In fungi, the cell wall is a dynamic structure that is essential for maintaining the osmotic balance of the cell, for creating and maintaining its shape during morphogenesis and providing it with protection from environmental stresses. In addition, in pathogenic fungi, the cell wall and especially cell wall proteins are known to play a key role in the relationship between the fungal cell and the host, contributing to host tissue adhesion and to immune response modulation (Plaine et al., 2008).

C. albicans cell wall (Figure 6) is a network composed predominantly of β -1,3-linked glucan (50%), containing some β -1,6-glucan branches (5%) that bind mannoproteins (Santos et al., 2000). The inner layer, containing chitin (10%), determines the shape and maintains stability of the cell, while the outer mannoprotein layer (40%) provide antigenic determinants and limit permeability (Chaffin et al., 1998; Eckert et al., 2007).

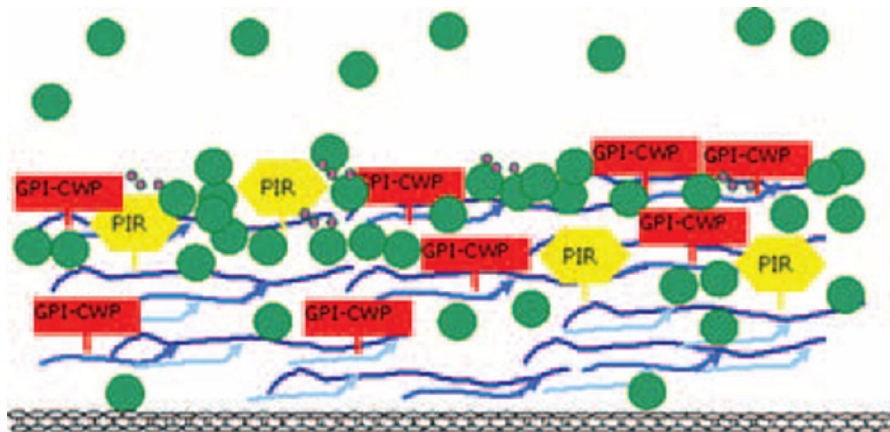


Figure 6. Schematic representation of major cell wall components.

The cell wall is external to the cell membrane (shown in black and white at the bottom). Labeled symbols: red rectangles, GlycosylPhosphatidylinositol cell wall proteins (GPI-CWPs); yellow hexagons, Proteins with Internal Repeats (PIR).

Unlabeled symbols: dark blue lines, β -1,3-glucan; medium blue lines, β -1,6-glucan; light blue lines, chitin; maroon circles, phosphomannolipid; green circles, unattached proteins found in the cell wall or in the medium (Chaffin W. L., 2008).

The major class of cell wall proteins has the sequence features of GlycosylPhosphatidylinositol (GPI)-anchored proteins, including an N-terminal signal sequence and a C-terminus containing a GPI anchor attachment site (x-site). GPI anchored proteins (GpiPs) are linked to the plasma membrane via a preformed GPI anchor that is added to the protein in the endoplasmic reticulum by a transamidase enzyme complex that removes the GPI anchor signal (Figure 7). Furthermore, most GPI anchored proteins are heavily glycosylated. In some fungi, several results suggest that a subset of GpiPs are cleaved from the membrane and translocated to the cell wall where they are linked covalently to β -1,6-glucan.

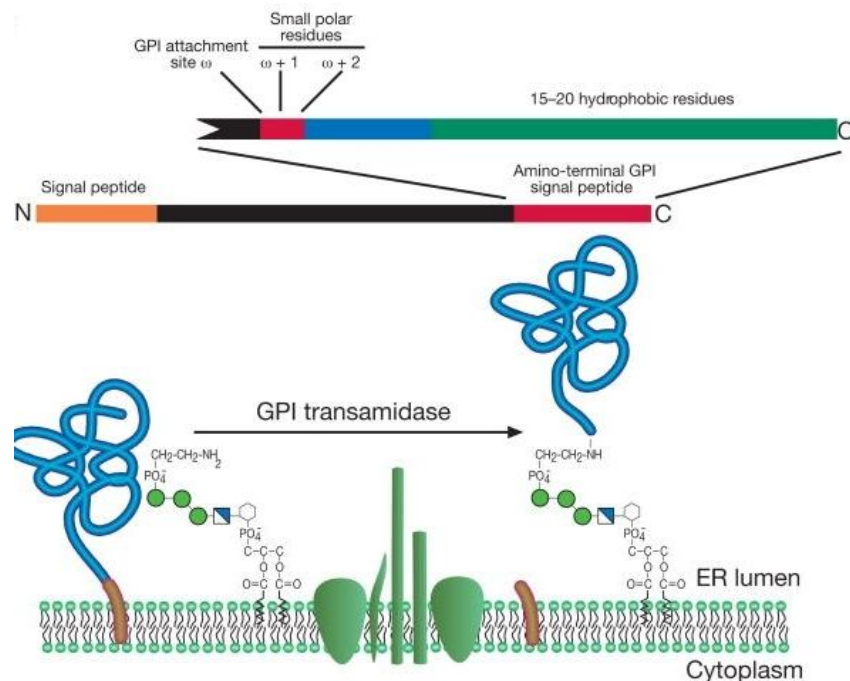


Figure 7. Features of GPI-anchored proteins and their processing by GPI transamidase.

GPI-anchored proteins have an amino-terminal signal peptide and a carboxy-terminal GPI-addition signal peptide (*top*) that is removed and directly replaced by a GPI precursor (*bottom*).

By comparing in silico predicted GpiPs reported in a number of studies, there is an estimate of 115 putative GpiPs in the *C. albicans* genome; almost twice as many putative GpiPs as in *S. cerevisiae*. As cell surface components, most are thought to be antigenic determinant proteins and virulence factors. To date, around 70 of them have been partially characterized by the generation of null mutants. Of these, 15 have been tested for virulence and 12 have shown a virulence defect in a mouse model of systemic infection (Plaine et al., 2008). Several functions are attributed to GPI proteins depending on the organism. Scanning the *C. albicans* genome at www.candidagenome.org revealed that no less than 65 of these 115 GPI anchored proteins are termed "PGA" (Predicted GPI Anchor). These proteins are not necessarily related in function or structure. They are grouped simply because they all share an N-terminal signal sequence and a C-terminal GPI anchor attachment site (Richard and Plaine, 2007). In characterization of cell wall proteins, the determination of whether such a protein is necessary for structural integrity or not is essential. This is usually achieved by creating a homozygous null strain for the protein in question and testing for sensitivity to a number of cell surface disrupting agents that target the cell wall or membrane. These included Calcofluor White (CFW), a fluorescent optical brightener with chitin-binding properties preventing chitin microfibril assembly, congo red, an inhibitor of glucan fiber assembly, and Sodium Dodecyl Sulfate (SDS), a detergent that compromises the integrity of the cell membrane. Since the cell wall is deposited above the cell membrane, any increased susceptibility to SDS implies a weaker cell wall. Antifungal susceptibility is studied by testing with caspofungin, a drug inhibiting the synthesis of β -1,3-glucan, the main component of the cell wall (Plaine et al., 2008).

1.5 Pga1

Pga1 is a yet-unknown uncharacterized short 132 amino acid putative GPI anchored protein of unknown function localized in cell wall and that has been shown to be upregulated under conditions of cell wall regeneration. The current study thus aims at characterizing the role of Pga1 as far as virulence in a mouse model of disseminated candidiasis, filamentation on solid and liquid filamentation and non-filamentation inducing media, susceptibility to cell surface disrupting agents such as SDS, calcofluor white, congo red, oxidative stress response to a potentially lethal dose of hydrogen peroxide, adhesion to human epithelial cells, and biofilm formation on polystyrene plastic surface, by creating a *pga1* heterozygote and homozygous null strains and comparing the phenotype of the mutant to the parental strain.

Most techniques to create a null mutant in *C. albicans* utilize a cassette known as the urablaster. The urablaster is a cassette that contains a *C. albicans URA3* marker flanked by bacterial *hisG* direct repeats. Upon subcloning of your gene of interest at the 5' and 3' end of the cassette, followed by transformation, the cassette should integrate at the gene of interest locus, effectively knocking it out. The advantage of such a technique is that the marker is recyclable since the *hisG* repeats can align and undergo intrachromosomal recombination popping out the marker. Recently, however, it has been shown that the *URA3* allele flanked by *hisG* repeats is not expressed to native levels resulting in a mutant phenotype (Sharkey et al., 2005). To avoid such complications, our null strain was generated by amplifying *URA3* and *HIS1* markers cassettes that included 100 bp homology regions from the 5' and 3' regions of *PGA1*. Since *C. albicans* is known to have a relatively high rate of homologous recombination (Magee et al., 2003), integration of these cassettes at the 2 *PGA1* loci created a *pga1* null strain. Such a technique is fast, cheap and straightforward with a high success rate.

Thus, one of the outcomes of this study is to further understand and characterize the mechanisms and factors employed by *C. albicans* that make it such a potent pathogen. In the long run, this study should contribute to the treatment and the well being of patients by generating possible antifungal drug targets.

Chapter 2

Materials and Methods

2.1 Deletion cassette and strain construction

The *Candida albicans* transforming plasmids, pABSK2 and pLHL, were utilized to amplify the *URA3* and *HIS1* deletion marker cassettes (Figure 8) respectively as described previously (Khalaf and Zitomer, 2001, Dib et al., 2008). Primers (Table 1) that included 100 bp sequences of the 5' and 3' *PGA1* open reading frame (ORF) were used to amplify either the *URA3* or the *HIS1* ORF resulting in cassettes that contain the marker gene, flanked by 100 bp *PGA1* sequences. The *Candida albicans* strain RM1000 (*ura3* Δ ::*limm434*/*ura3* Δ ::*limm434bis1*::*bisG*/*bis1*::*bisG*, Negredo et al., 1997) was used in this study and was transformed by the constructed *URA3* marker cassette. By homologous recombination, the marker cassette integrated at the *PGA1* locus creating a *pga1*::*URA3/PGA1* heterozygote strain. For integration verification, a *PGA1* primer upstream of the integration site, and an internal *URA3* primer were utilized for PCR amplification on genomic DNA extracted from colonies grown on selective media lacking uridine. The *pga1*::*URA3/pgal*::*HIS1* null mutant strain was generated from the heterozygote strain by transforming cells with the constructed *HIS1* marker cassette. Deletion of the second allele was verified by PCR amplification utilizing the same upstream *PGA1* primer and an internal *HIS1* primer on genomic DNA extracted from colonies grown on selective media lacking uridine and histidine. Furthermore, confirmation of the *pga1* deletion was achieved by PCR

amplification using a set of primers that hybridize to internal sequences of the *PGA1* allele (Figure 9).

Table 1: Primers used throughout this study.

No.	Primer	Sequence	5' primer hybridization locus
1	<i>PGA1-URA3DelF</i>	5'TAATAAGATTATTTGAGTTTCAAACCAGTT TGATACATTTTTTCATTAGAATATAGATTTAA AGTTCCGTCCATTTGTTTATTGAGTCGTACA TTTTCAAAGATGGAATTGATTTGGATGGTA TAAACGG 3'	- 413
2	<i>PGA1-URA3DelR</i>	5'TAATGTTGAAAACCTAGTCAGCCAGCTGT ATCCTCCCATTTTATAATAAACCTAAAAT CAAACAATACCACCAACAATGCCTGTGTA CAAACCTCAATGCCGACTGTCATGATTTTC TAGAAGGACCAC 3'	+ 953
3	<i>PGA1-HIS1DelF</i>	5'TAATAAGATTATTTGAGTTTCAAACCAGT TTGATACATTTTTTCATTAGAATATAGATTTA AAGTTCCGTCCATTTGTTTATTGAGTCGTAC ATTTCAAAGATCAGAAGTTAGTAGTAAC AATTTGG 3'	- 320
4	<i>PGA1-HIS1DelR</i>	5'TAATGTTGAAAACCTAGTCAGCCAGCTGT ATCCTCCCATTTTATAATAAACCTAAAAT CAAACAATACCACCAACAATGCCTGTGTA CAAACCTCAATGCCGAATAATATTTATGA GAAACTATCACTTC 3'	+ 926
5	<i>PGA1DelVerF</i>	5'CAAACGTCAAATTGCAATAAAATAAGC CCAG 3'	- 170
6	<i>PGA1InternalF</i>	5'CGTGTTATTTGGCATGTGTATTTGCATTG 3'	+ 40

7	<i>PGA1</i> InternalR	5'GCTTGGGGCTATGTTAGCATTGGT 3'	+ 300
8	Actin F	5'GTTGCTGCTTTAATTATCGATAACGG TTCTGG 3'	+ 674
9	Actin R	5'GGTGAACAATGGATGGACCAGATTCGTC 3'	+ 1776
10	<i>URA3</i> verification R	5'CGGTCTGGTAAATGATTGACTAAATCCTCC 3'	+ 30
11	<i>HIS1</i> verification R	5'CGGTCTGGTAAATGATTGACTAAATCC 3'	+ 30

Sequences listed mentioning its hybridization position at the locus 5' end. Negative digits refer to promoter sequences. Positive digits refer to the ORF sequence relative to the transcription start site (+1).

2.2 Media preparation and cell growth

All strains were cultured without selection on rich potato dextrose agar (PDA) medium (Himedia Laboratories, Marg, Mumbai, India). For selective growth, yeast nitrogen base (YNB) synthetic medium (Fluka, Switzerland) was used lacking specific nutrients (Kaiser et al. 1994). All media used in the assay was supplemented with 50 µg/ml uridine and histidine.

For filamentation assays on solid media, cultures were grown to a density of 3×10^7 cells/ml, washed, and resuspended in PBS buffer at that same density. Samples with a volume of 5 µL were spotted on PDA, PDA with 20% fetal bovine serum (FBS, BioWhittaker Incorporated, Walkersville, MD, USA), medium M199 with Earle's salts, L-glutamine buffered to pH 4 or pH 7.5 (Sigma-Aldrich Corp. St. Louis, MO, USA), or Lee's media buffered to pH 6.8. Cells were incubated for 3-4 days at 30°C and 37°C and monitored daily.

For filamentation assays on liquid media, cells were inoculated in the same media mentioned above for 12 hr. Cell morphologies were examined microscopically after a period of 3 to 4 hr and after 12 hr.

Images were generated utilizing an Olympus E330-ADU-1.2x stereomicroscope for colony morphology. An Olympus CX41 was used for cell imaging coupled with a Sony DSC-S40 digital camera.

2.3 Cell culture

The human colon adenocarcinoma cell line HT-29 ATCC number HTB-38 was utilized for adhesion studies. Cells were grown in RPMI 1640 medium containing 25 mM Hepes and L-glutamine supplemented with 10% FBS, 100 µg/ml of streptomycin and 100 U/ml of penicillin in a 5% CO₂ environment.

2.4 Oxidative stress assays

Oxidative stress was performed as described previously (Dib et al., 2008). Parental, heterozygote, and null strains were grown in rich PDB media to exponential phase, adjusted to a cellular density of around 3×10^6 cells/ml, and treated for 180 min with 25 mM or 50 mM hydrogen peroxide. Cellular viability was monitored at 30 min intervals by optical density measurements. Untreated strains were used as controls.

Furthermore, the same exponential phase cultures were treated with 10, 25, or 50 mM hydrogen peroxide concentrations for one hr, after which 10 fold serial dilutions were made and 5µl of cells were spotted on PDA (Pedreno et al., 2007).

2.5 Cell surface disrupting agents

Strains were grown on rich PDB media and their cellular density was adjusted to 5×10^5 cells/ml. Serial dilutions were performed (10^5 to 10^1 cells/ml) and a sample volume of 5µl was spotted on PDA plates in the presence of one of the following cell surface disrupting agents: SDS at a concentration ranging from 0.02%-0.05%, calcofluor white at a concentration ranging from 50-300 µg/ml,

and congo red at a concentration ranging from 20-75 $\mu\text{g}/\text{mL}$. Plates were incubated at 30 °C and monitored for 4 days. Growth on PDA plate lacking the disrupting agents was also performed and used as a growth control (Plaine et al., 2008).

2.6 Antifungal susceptibility assays

The antifungal susceptibility test was determined using the Epsilon meter test (E-test) method on RPMI 1640 medium (supplemented with L-glutamine and 165 mM MOPS, BioWhittaker). E-test strips of amphotericin B (AP), caspofungin (CS), and ketoconazole (KE) were supplied by AB Biodisk (Solna, Sweden). Inoculum preparation, inoculation, and strip application were performed according to the manufacturer's recommendations. Minimal inhibitory concentration (MIC) readings were done after 48-hr incubation at 35°C. As recommended by the manufacturer, amphotericin B MICs were read as the lowest concentration on the E-test strip where there was 100% growth inhibition; at the point where the border of the elliptical inhibition zone intersects the strip. On the other hand, caspofungin and azoles MICs were read as the lowest concentration at which there was 80% inhibition or where there was a significant decline in growth. The MIC interpretive breakpoint criteria that were used in this study: (R), $\geq 1 \mu\text{g}/\text{mL}$ for ketoconazole; (R), $\geq 0.38 \mu\text{g}/\text{mL}$ for amphotericin B; and (S), $\leq 1 \mu\text{g}/\text{mL}$ for caspofungin (Dib et al., 2008). The *C. albicans* CLSI reference strain ATCC 90028 was used for quality control. All the obtained MIC's fell within the control limits. MIC range for ATCC 90028 is provided by the E-test manufacturer.

2.7 Adhesion assay

Adhesion to epithelial cell line HT-29 was performed as previously described (Sharkey et al., 1999). *C. albicans* strains (~100 cells) were added to 6 well microtiter plates containing human epithelial cells and allowed to incubate for either 90 min or 180 min. Non-adherent cells were aspirated by washing with PBS and the wells were overlaid with warm agar. After 24 hr growth, the colonies were counted and compared to controls on sabouraud agar plated. Adhesion was expressed as a percentage of total cells added. Assay was repeated twice. Averages are shown.

2.8 Biofilm formation

Biofilm formation on polystyrene plastic microtiter plates was done as described previously (Peeters et al., 2008). 5×10^6 cells were added to a flat bottomed microtiter well pretreated overnight with 5% serum at 4 °C and incubated in a shaker (75 rpm) at 37 °C for 2 hr to allow adhesion. Non-adherent cells were rinsed away with PBS and plates were re-incubated for 48 hr and rinsed again. Methanol fixation followed by addition of a 0.2% crystal violet solution for 20 min was performed. Unfixed crystal violet was washed away and bound crystal violet was released by acetic acid treatment. Absorbance of released crystal violet was measured at 590 nm. A negative control lacking adherent cells was also performed. Assay was repeated twice. Averages are shown.

2.9 Disseminated candidiasis virulence assay

3×10^7 freshly grown *C. albicans* cells of each strain were washed, resuspended in PBS solution, pH 7.5 and injected into female mice (Lebanese American University stock); 7 for the wild type and 14 for the null mutant; weighing 20-30 grams via the lateral tail vein. Mice were fed with food and water *ad libitum* and monitored 3 times daily for survival over a period of 20 days.

2.10 Statistical analysis

For the virulence assay, the Kaplan–Meier survival curve and the log Rank test were performed on the data collected using the SPSS computer software (SPSS Inc., USA). For adhesion assay, P-value was obtained by 2 factor analysis of variance with the interaction term. For biofilm formation assay, statistical analysis was done using the multiple comparison technique L.S.D (least significant difference). P values below 0.05 were deemed significant.

2.11 Homology assessment

A search for possible Pga1 homologues was done by BLASTing the Pga1 sequence onto the *C. albicans* database at www.candidagenome.org. The closest *C. dubliniensis* orthologue was retrieved from that same database. BLASTing the Pga1 sequence onto the *Saccharomyces cerevisiae* genome (www.yeastgenome.org) was done to determine the closest *S. cerevisiae* orthologue.

2.12 Protein modeling

The sequence of Pga1 was retrieved from the previously mentioned *Candida* genome database and entered into these databases, <http://www.ebi.ac.uk/Tools/InterProScan/> and <http://www.cbs.dtu.dk/services/TMHMM/> to determine possible protein domains or transmembrane regions.

Chapter 3

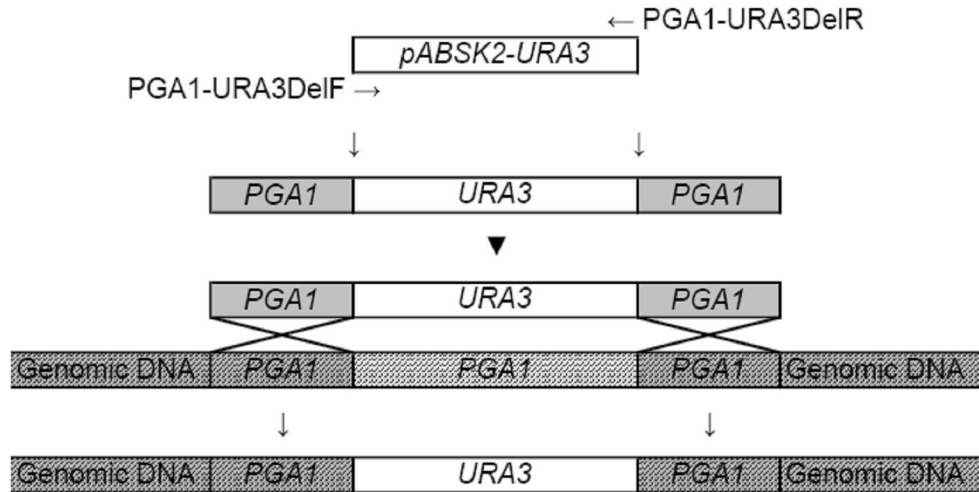
Results

3.1 Verification of integration

Transformation followed by homologous recombination of a *URA3* cassette of 1566 bp followed by a *HIS1* cassette of 1466 bp deleted the two alleles of *PGA1* respectively producing a *PGA1/pgal::URA3* heterozygote and a *pgal::URA3/pgal::HIS1* homozygote null strains (Figure 8).

Proper integration was verified by amplifying a DNA fragment of 580 bp and 500 bp for the *URA3* cassette and the *HIS1* cassette respectively by the use of a *PGA1* forward primer located outside of the integration site and a *URA3* or *HIS1* internal reverse primer. Confirmation of the absence of a *PGA1* allele in the null mutant was achieved by amplifying with *PGA1* internal primers (Figure 9).

(A)



(B)

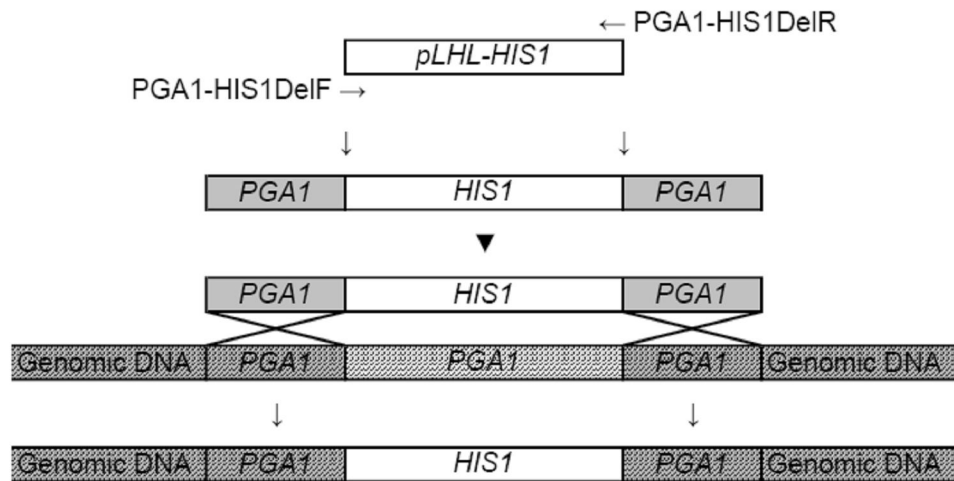
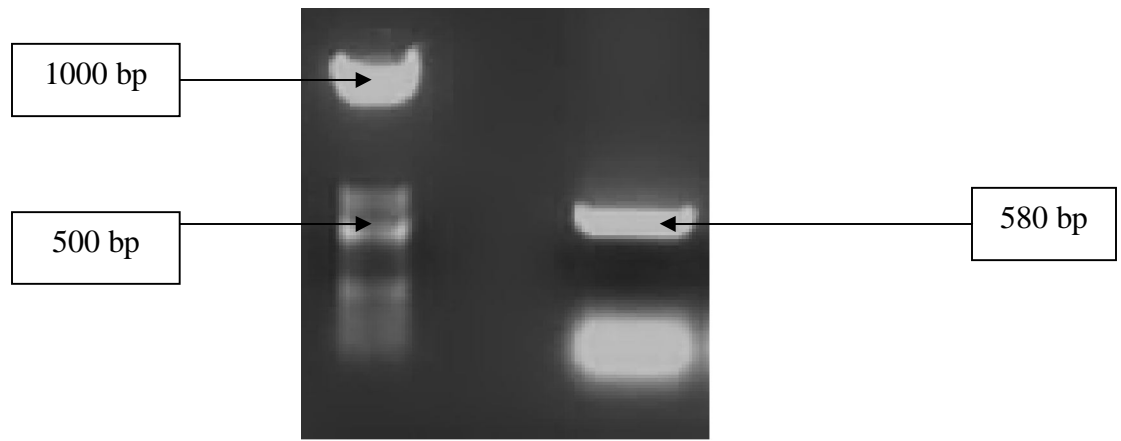


Figure 8. *PGA1* deletion strategy.

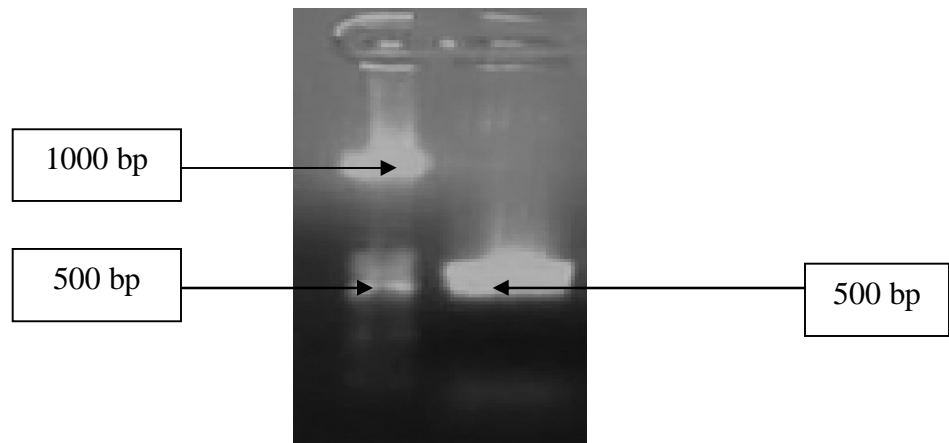
Primers with flanking *PGA1* sequence were used to amplify the marker genes generating integration cassettes. Upon transformation, homologous recombination at the 5' and 3' flanking regions should occur generating functional marker genes and *pga1* mutant alleles.

(A) *URA3* recombination scheme

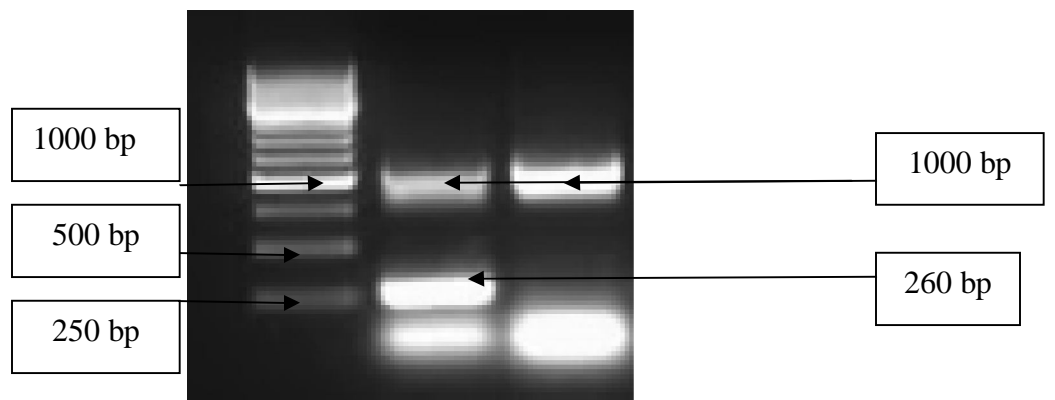
(B) *HIS1* recombination scheme



(A) M



(B) M



(C) M 1 2

Figure 9. Deletion verification.

(A) Presence of a 580 bp band confirming the generation of the *PGA1/pgal1::URA3* heterozygote by the integration of the URA3 fragment.

(B) 500 bp fragment confirming the integration of the *HIS1* cassette and the generation of the *pga1::URA3/pgal1::HIS1* null.

(C) Upper 1 kb bands in both lanes are for actin gene control; Lane 1: the presence of a 260 bp band in the heterozygote from A confirms the presence of a *PGA1* allele, while in Lane 2 the lack of an internal PCR fragment confirms its absence in the null strain

Lane M: Marker DNA ladder

3.2 Protein Domain Modeling

The 132 amino acid sequence of Pga1 was entered into both databases to determine possible protein domains and transmembrane regions. As can be seen in Figure 10, Pga1 consists of possibly three transmembrane domains, a short region protruding into the cell wall and a long cytoplasmic domain.

(A)

SignalIP		signal-peptide
Tmhmm		transmembrane_regions

(B)

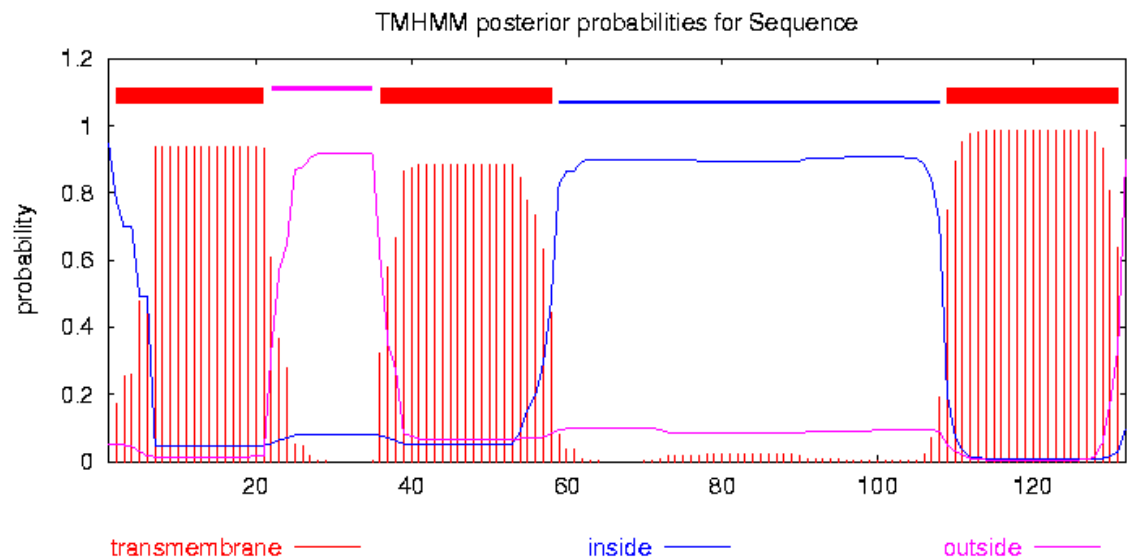


Figure 10. Pga1 protein domain.

A) The InterProScan program predicted three transmembrane regions for Pga1, including an N-terminal signal peptide.

B) The TMHMM service confirmed this finding and suggested that in addition to these transmembrane regions, the protein consists of one main intracellular domain with another domain protruding into the cell wall from the membrane.

3.3 Filamentation assay

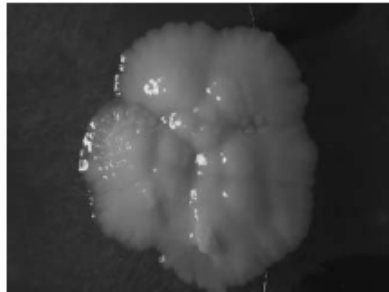
On solid media:

The wild type RM1000 + pABSK2, *PGA1/pgal::URA3* heterozygote and *pgal::URA3/pgal::HIS1* null were grown on PDA plates for 4 days at 30°C and 37°C. As can be seen in Figures 11, the null mutant was over-filamentous as compared to the wild type strain with the heterozygote mutant showing an intermediary phenotype. No differences in growth were observed in cultures on medium M199 (pH 4 or pH 7.5), or Lee's media (pH 6.8) (data not shown).

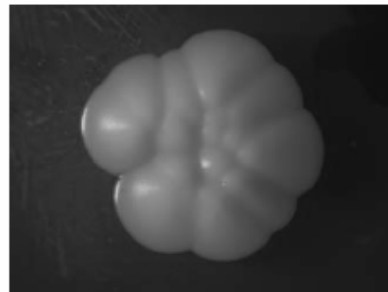
In liquid media:

All 3 strains were grown in PDB at 30°C overnight. A similar discrepancy in phenotypes was noted as above (Figure 11).

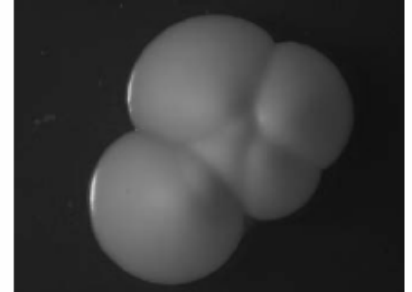
PDA 30°C



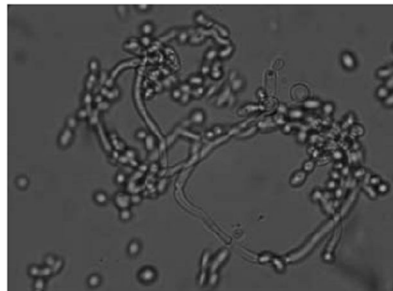
Null Mutant



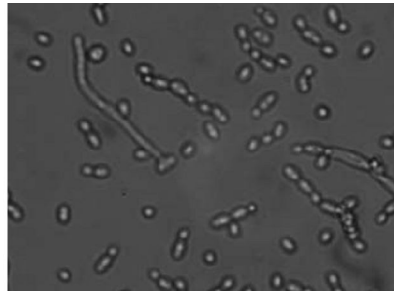
Heterozygote Mutant



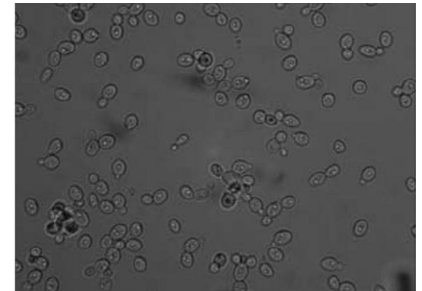
Wild Type



Null Mutant

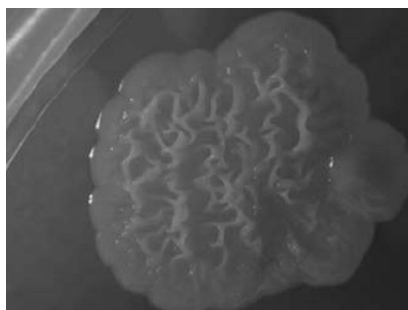


Heterozygote Mutant



Wild Type

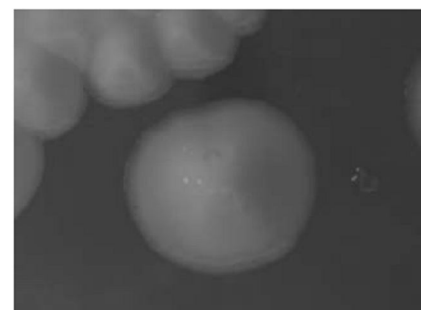
PDA 37°C



Null Mutant



Heterozygote Mutant



Wild Type

Figure 11. Growth on PDA media.

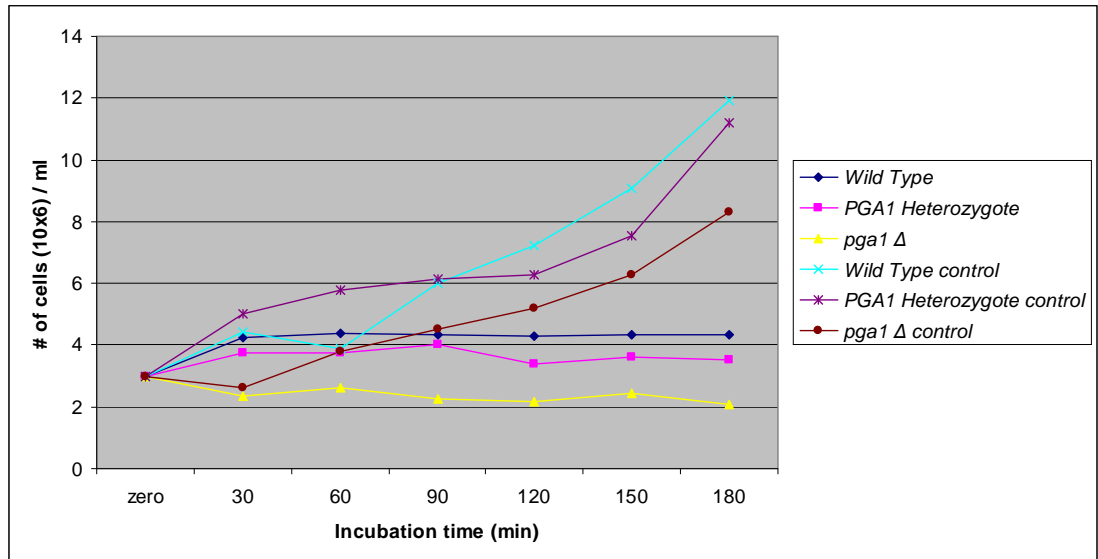
Strains were grown on liquid and solid PDA as described above and photographed at 20X magnification (solid), 1000X (liquid).

Note the increased filamentation in the null as compared to the wild type strain after 4 days of growth (solid) and 12 hr (liquid) at both 30 and 37°C.

3.4 Oxidative stress assays

The oxidative stress assay was performed by two different methods. The first consisted of treating cells with different concentrations of hydrogen peroxide (25 mM and 50 mM) and monitoring cell growth spectrophotometrically at 30 min intervals for 180 min. At 50 mM H₂O₂ concentration a very slight decrease in cell number was observed in the mutant as compared with the parental strain (Figure 12 A). In another assay, all three strains were subjected to various hydrogen peroxide concentrations (10, 25 and 50 mM) for 1 hr and then spotted onto PDA plates after serial dilutions. The homozygous mutant strain showed a marked decrease in resistance to 50 mM hydrogen peroxide concentrations in comparison to the heterozygote and wild type strains (Figure 12 B).

(A) 50 mM H₂O₂



(B) 50 mM H₂O₂

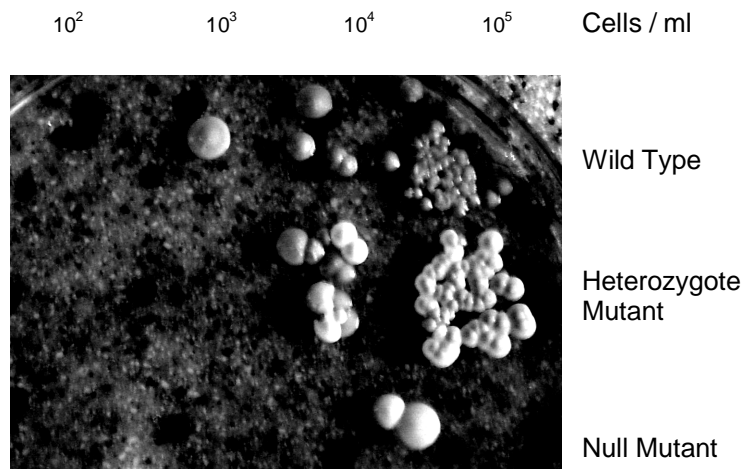


Figure 12. Oxidative stress challenge.

(A) Cells were grown in the presence of 50 mM H₂O₂ and monitored at 30 min intervals. Untreated cells serve as controls. Note the slight decrease in cell number in the mutant over time.

(B) Cells were grown in PDB and challenged with 50 mM H₂O₂ for 1 hr after which serial dilutions were made and cells plated on PDA. Note the marked decrease in resistance to oxidative stress in the mutant.

3.5 Cell surface disrupting agents

The three strains were spotted onto PDA agar at five different concentrations and subjected to various concentrations of cell wall disturbing agents as described in the Materials and Methods. Figure 13 shows that the null strain was found to be more sensitive to calcofluor white but more resistant to SDS with no observable phenotypic difference with the wild type upon congo red exposure.

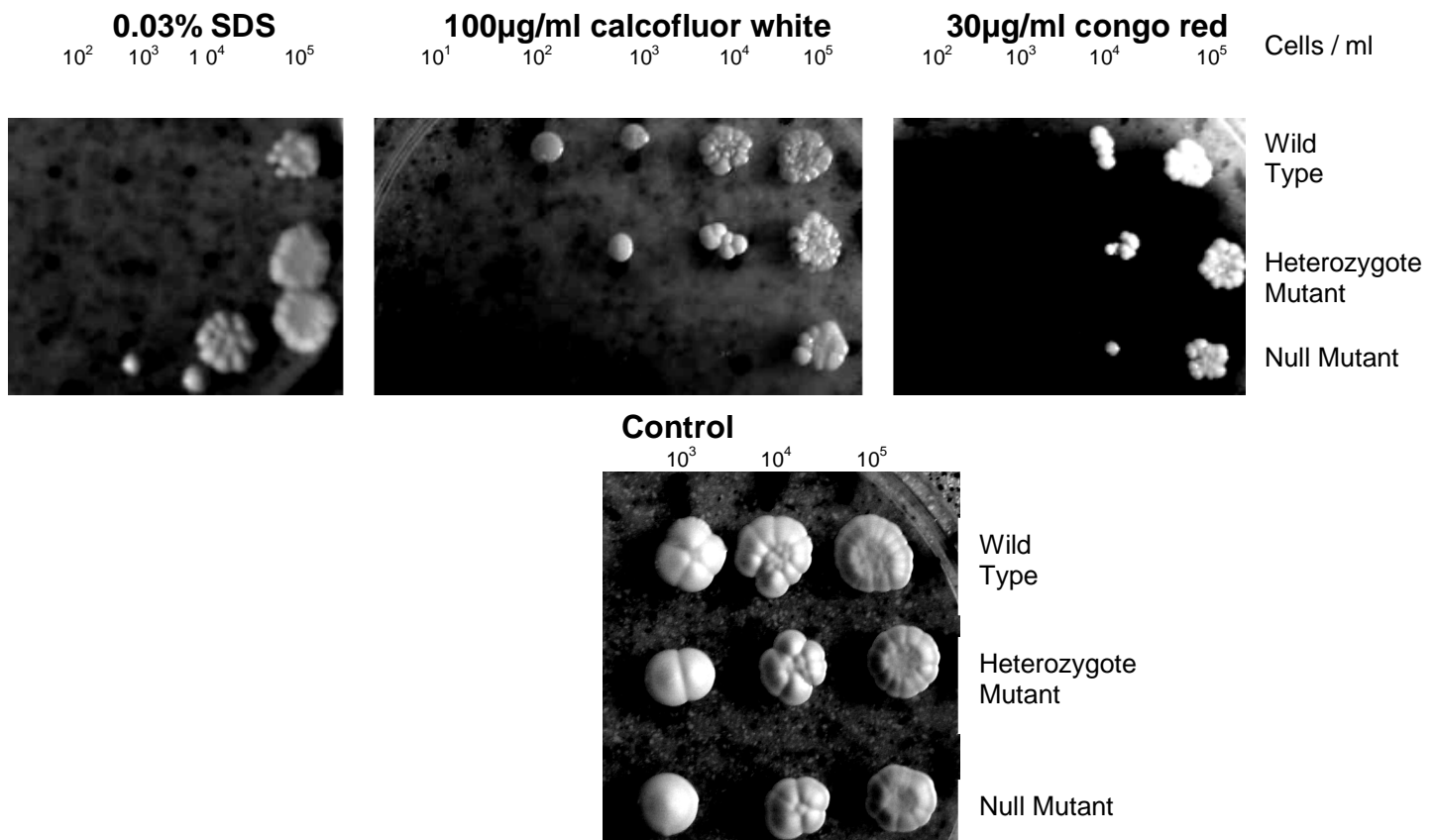


Figure 13. Susceptibility to cell surface disrupting agents.

Cells spotted at various concentrations as described in the Materials and Methods and grown in the presence of cell surface perturbing agents at various concentrations.

Note the increased sensitivity of the null to calcofluor white as opposed to an increased resistance to SDS.

3.6 Antifungal sensitivity

Sensitivity to ketoconazole, amphotericin B and caspofungin was determined for the 3 strains using the E-test method after 24 hr of incubation. As can be seen in Table 2, no significant discrepancy was observed between the strains implying no altered sensitivity to any of the three antifungal agents.

Table 2. Antifungal susceptibility.

Anti-fungal agent	Wild type	<i>PGA1/pga1</i>	<i>pga1/pga1</i>
Amphotericin B	0.75	0.5	1
Caspofungin	0.19	0.25	0.125
Ketoconazole	0.032	0.016	0.032

All three strains were assayed for antifungal susceptibility by the E-test method. Numbers represent MIC's in µg/ml. No significant difference between the mutant and wild type strain was observed.

3.7 Adhesion assay

The wild type, heterozygote and null mutant strains were incubated with human epithelial cell line Ht-29 for 90 and 180 min. A dramatic and statistically significant decrease (p less than 0.001) was observed in the adhesion of the *pga1* null strain compared to the wild type where the heterozygote strain showed an intermediary phenotype at both incubation times (Figure 14).

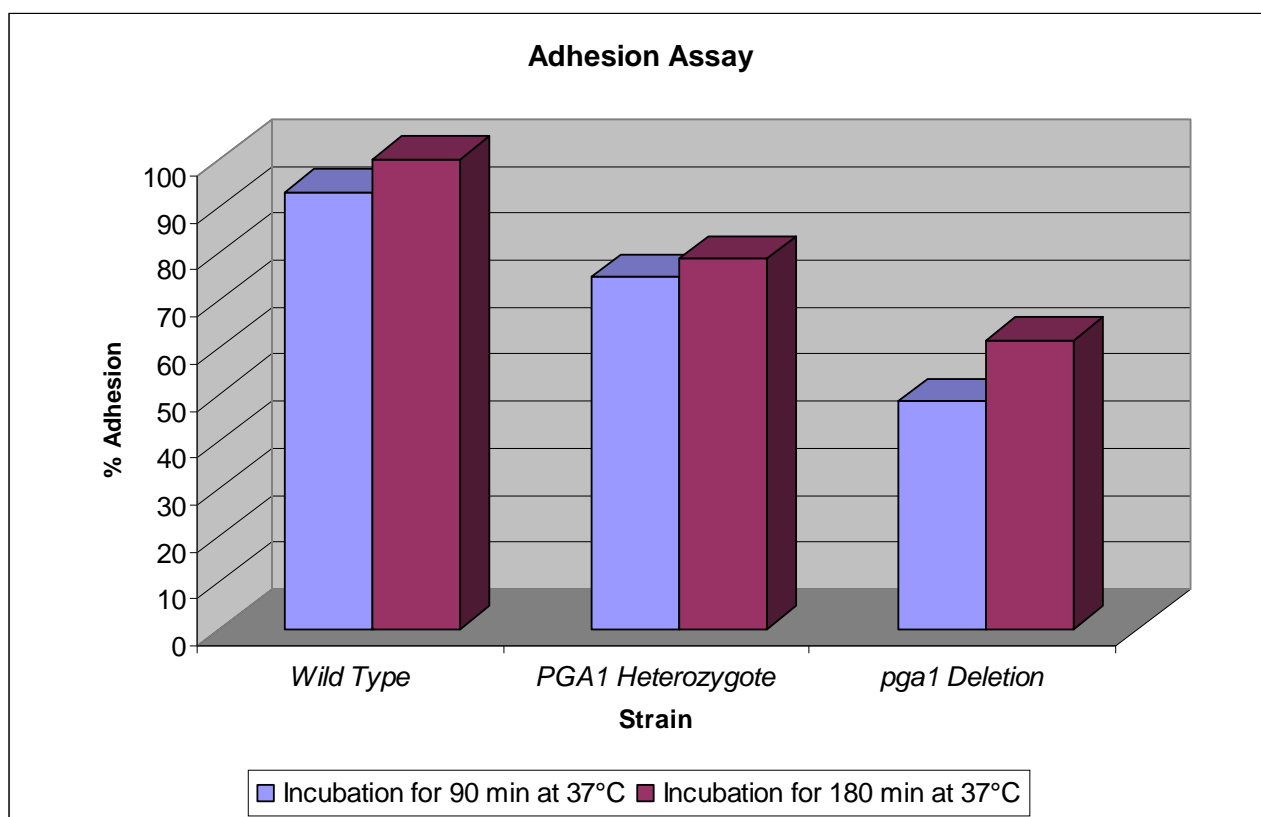


Figure 14. *C. albicans* adhesion to human epithelial cell line HT-29.

Around 100 cells of all three *PGA1* strains were incubated with human epithelial cells in 6-well microtiter plated for 90 and 180 min as described in the Materials and Methods. The percent adhesion was expressed as percent adherent cells versus total cells added.

Note the statistically significant (p less than 0.001) and dramatic decrease in adhesion of the *pga1* null strain compared to the wild type, with the heterozygote strain exhibiting an intermediary phenotype.

3.8 Biofilm formation

The three above mentioned strains were allowed to form biofilm on polystyrene wells as described in the Materials and Methods. Both mutants were statistically significantly defective in biofilm formation in comparison to the wild type (p less than 0.001 for both mutants compared to the wild type). A negative control whereby no cells were added was also performed (Figure 15).

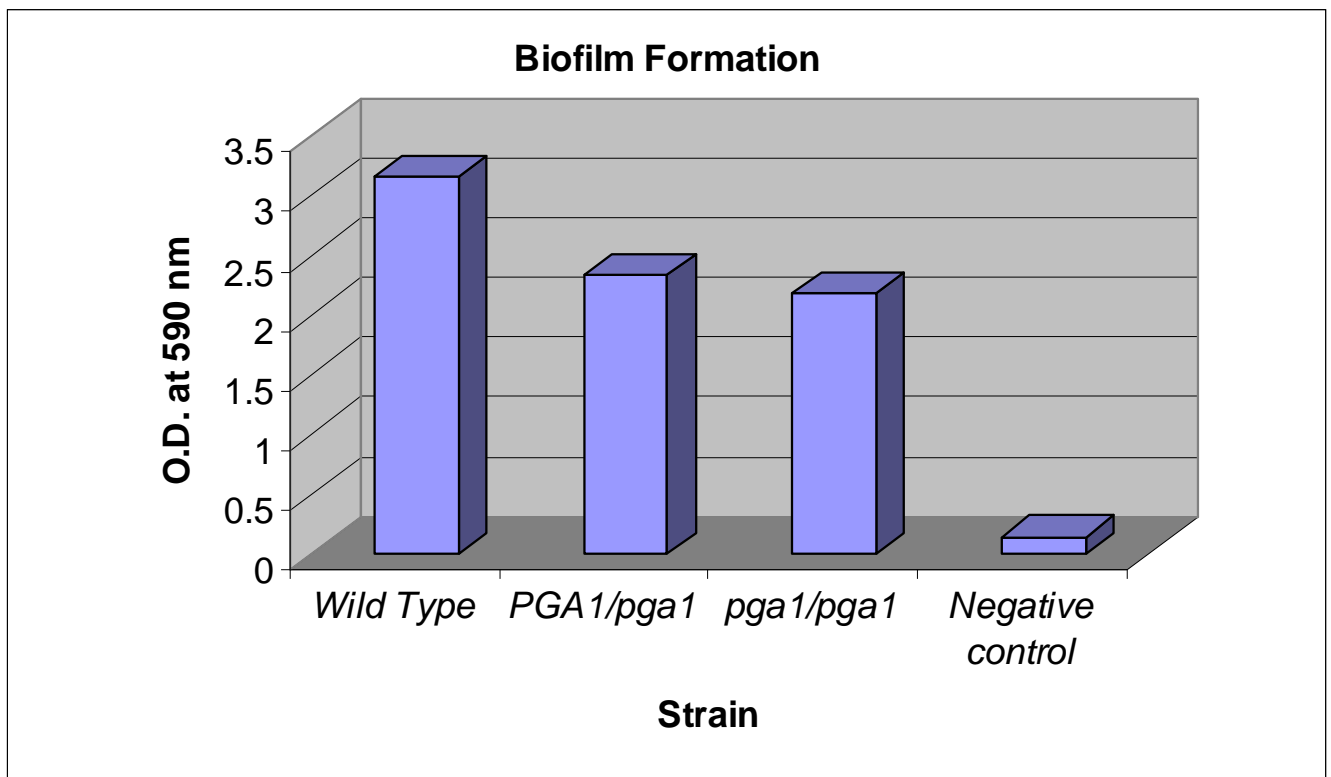


Figure 15. Biofilm Formation.

Biofilm formation on polystyrene plastic microtiter plates done as described previously. Absorbance of released Crystal Violet was measured at 590 nm. A negative control lacking adherent cells was performed.

Note the decreased crystal violet concentration (33%) in heterozygote and null mutant strains in comparison with wild type strain corresponding to a decrease in biofilm formation.

3.9 Murine model of disseminated candidiasis

BALB/c mice were injected in the tail vein with 3×10^7 *C. albicans* cells of either the wild type or the null mutant strain. As can be seen in Figure 16, all mice injected with the wild type strain were killed while around 14% of mice injected with the mutant survived. This slight discrepancy was however not statistically significant ($p=0.8$) Thus, the homozygote null strain might be similar to the wild type as far as virulence is concerned.

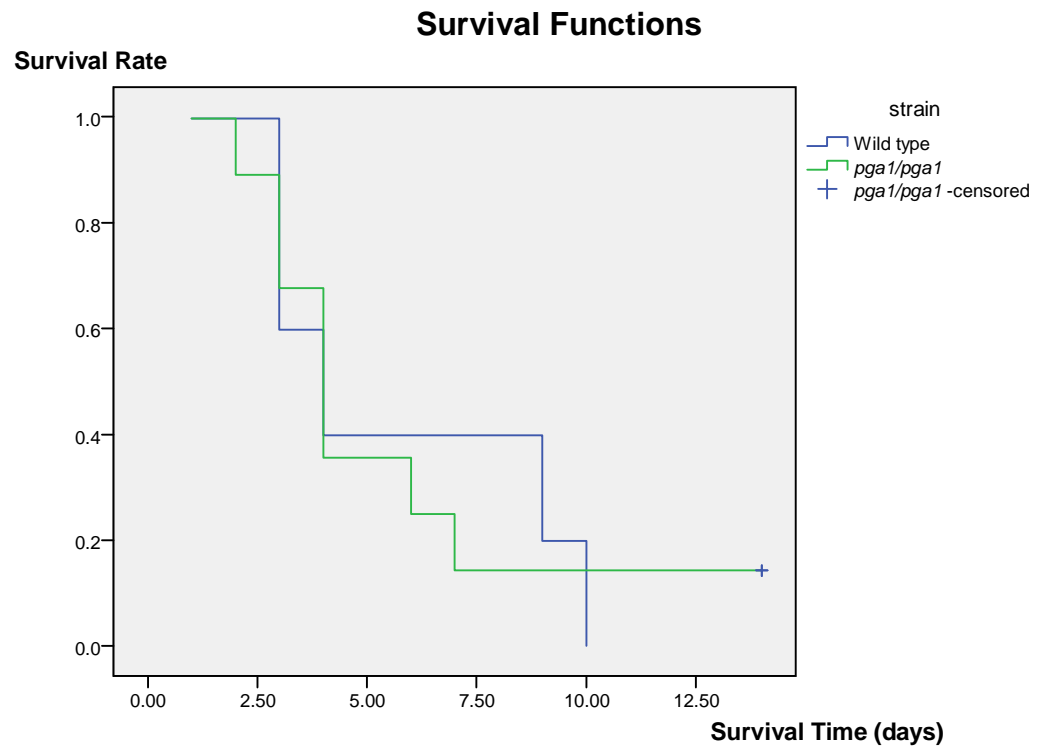


Figure 16. Disseminated candidiasis virulence assay.

Disseminated candidiasis experiment was performed on female BALB/c mice; 7 for the wild type and 14 for the null mutant as described in Materials and Methods. For statistical analysis, the Kaplan–Meier survival curve and the log Rank test was performed.

Note the slight but not significant difference in survival between the two strains ($p=0.8$).

Chapter 4

Discussion

The cell wall of many fungi including *C. albicans* is a complex entity consisting mainly of β -1,3-linked and β -1,6-glucan in addition to chitin and mannoproteins which determine surface properties, enabling *C. albicans* cells to interact and adhere to host tissues (Chaffin et al., 1998). As antigenic determinants, *Candida albicans* cell wall proteins in general, and GPI anchor proteins in particular, can be possible targets for novel antifungal agents and as such research is increasingly focusing on characterizing them as they have been known to play a role in virulence and filamentation.

Based on the literature in www.candidagenome.org, the PGA protein family comprises not less than 65 members. Most are uncharacterized proteins sharing no real sequence similarity amongst them besides the N-terminal signal peptide and C-terminal GPI anchor site. Some are actually aliases of characterized ORFs, such as Pga2 and Pga3, which are more commonly known as *SOD4* and *SOD5* coding for superoxide dismutases (Martchenko et al., 2004), or Pga47, known as Eap1, which is necessary for adhesion and biofilm formation (Richard and Plaine, 2007).

Furthermore, no *PGA1* homologue is found within *C. albicans*. Upon BLASTing the amino acid sequence of Pga1 onto the *Saccharomyces cerevisiae* database (www.yeastgenome.org) however, we found a protein, Kre1, with strong amino acid similarity to Pga1 and as such a possible *S. cerevisiae* orthologue. Kre1 has been actually shown to be involved in β -glucan assembly (Lesage and Bussey, 2006). The *Candida* database also revealed a possible orthologue in *Candida*

dublinsiensis (a very close relative of *C. albicans*). However this protein has not yet been characterized to give us any insight as to the role *pga1* plays in *C. albicans*.

The fact that *PGA1* has been shown to be induced during cell wall regeneration might imply a role in cell wall rigidity and stability of the organism, and, as a GPI-anchored protein, it might also play a role in virulence or filamentation (Castillo et al., 2006). We thus decided to characterize Pga1 by generating a *pga1* null strain.

In addition, C. Ojaimi (personal communication) has generated a *PGA1* revertant strain. In a revertant strain, the deleted allele is replaced to confirm that any phenotypic discrepancy between the mutant and the wild type is in fact due to the deletion and not to another secondary mutation. The revertant strain has confirmed the above mentioned phenotypes.

Our null strain showed many phenotypic differences compared to the parental wild type strain. One pertains to the general oxidative stress response. Two experiments were performed to address the issue. The reason behind performing two different experiments is because initially we measured cell viability to a hydrogen peroxide challenge spectrophotometrically. However, as the cellular optical density did not vary much upon hydrogen peroxide addition, we could not determine whether the cells were dead but intact, or alive but not dividing. To address this issue, we decided to perform the spotting test. Our data showed that Pga1 is necessary for proper oxidative stress response even though it is not known to play a role in the Hog1 general stress response pathway activated upon oxidative stress (Enjalbert et al., 2006). One explanation would be an indirect effect through a weakening of the cell surface in the mutant. Such reasoning also explains the increased susceptibility on the mutant to calcofluor white, a cell wall disrupting agent that prevents chitin microfibril assembly (Herth 1980). This theory is supported by the fact that *PGA1* is induced during cell wall regeneration and thus plays a role in cell wall stability

and rigidity (Castillo et al., 2006). Interestingly, this susceptibility was not evident with caspofungin or Congo red, two other cell wall disrupting agents, which inhibit β -1,3-glucan synthesis and glucan fiber assembly (Kopecka et al., 1992) respectively. The calcofluor and caspofungin data suggest that Pga1 might not be necessary for glucan microfibril biogenesis and assembly as inferred from homology assessment but rather for proper chitin microfibril assembly and maintenance.

An interesting and initially unexpected phenotype was the increased resistance to SDS, a cell membrane solubilizing agent. The questions that arise is why would a cell wall protein dramatically destabilize the cell membrane to such an extent and second, why is the phenotype one of increased resistance rather than increased sensitivity which is what one might expect after mutating a cell surface protein. The first question can be answered by the protein domain modeling data. The data showed the Pga1 probably consists of no less than three transmembrane domains. Based on this information, it should come as no surprise that the cell membrane became affected by the deletion. The second question however is more difficult to answer and assumes that the cell has upregulated key cell surface response elements to compensate the deletion. In fact, Plaine et al. (2008) has shown that one key response element to cell wall mutations that destabilize the cell surface is thickening of the cell wall through increased chitin deposition. Whether there is an increase in chitin levels in our mutant remains to be seen. However, the oxidative stress data suggests otherwise. Upregulation of cell surface proteins might have occurred that decreased SDS permeability without decreasing H_2O_2 permeability. Alternatively, the mutants might have destabilized and altered the cell wall architecture thus preventing anchoring of SODs on the cell surface that are necessary for proper oxidative stress response.

Another interesting phenotype of the mutant is its over-filamentation compared to the parental. The reason for this is yet to be determined. Again, however, whether the cell compensates a *pga1* deletion and responds by up regulating other cell surface components and filamentation induced genes is possible.

Our adhesion assays to human epithelial cells showed the mutant to bind cells 50% less than the wild type after 90 min incubation. This phenotype was not simply due to a delay in adhesion but to an adhesion defect since incubating for an extended period of time did not abolish the phenotype. Pga1 is not assumed to be a cell wall adhesin as it does not display similarity in sequence to other adhesins, or consist of an immunoglobulin-like domain followed by serine-threonine-rich conserved repeat regions, characteristic of many adhesins (Rauceo et al., 2006). The observed phenotype is thus indirect. By altering the morphology, composition, and structure of the cell surface, a *pga1* deletion might prevent proper anchoring and positioning of adhesins on the cell surface. The biofilm data confirms the adhesion defect as our mutant could only adhere to polystyrene plastic 65% as well as the wild type. Since adhesion to plastic is a precursor to biofilm formation, one would definitely expect a defect in adhesion to be translated as a defect in biofilm formation. In this sense, a *pga1* null strain shows a similar phenotype to a deletion of another GPI-anchored protein, Pga47, which has also been shown to be necessary for proper adhesion and biofilm formation and which, interestingly is induced during cell wall regeneration. Richard and Plaine, 2007 theorized that deletion of *pga47* produced a weak, unstable and non rigid cell wall that could not handle the formation of biofilm layers.

Finally, the virulence data indicated the possibility that our *pga1* null strain was slightly more attenuated in virulence, a phenotype to be expected bearing in mind the defect in adhesion. Many cell wall mutations have shown that a lack of adhesion is mirrored as a lack in virulence. The logic behind such argument is

simple, if a pathogen cannot adhere, it cannot invade tissues and organs to cause systemic infection and death (Karkowska-Kuleta et al., 2009). It is important to note however that the difference in virulence between the parental and null strains did not reach statistical significance and thus a larger more statistically significant number of mice should be infected in future experiments to clearly address this issue.

Chapter 5

Conclusion

Our preliminary characterization of Pga1 revealed that the protein is necessary for proper cell wall integrity, adhesion and biofilm formation. In addition, the mutant strain showed hyperfilamentation and an increase in resistance to oxidative stress. More experiments are needed to fully address the role Pga1 plays, notably microarray analysis to determine which filamentation inducing genes are up or down-regulated in response to this deletion, matrix-assisted laser desorption/ionization-time of flight mass spectrometry (MALDI-TOF MS) to determine changes in the cell wall proteome that might have occurred to compensate for such a deletion. Furthermore, cell wall thickness and especially chitin content should also be determined to reveal whether in a *pga1* null mutant, chitin assembly is disrupted as suggested by the calcofluor white assay, or whether on the contrary, the cell compensates the mutation by upregulating chitin content.

Chapter 6

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