LEBANESE AMERICAN UNIVERSITY

Cell Surface Proteome Characterization of the *Candida albicans DSE1* mutant

By

Racha M. Zohbi

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

School of Arts and Sciences

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LAU School of Arts and Sciences

Lebanese American University
School of Arts and Sciences – Byblos Campus

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Division: **Natural Sciences Department**

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Committee Member	Dr. Georges Khazen	[redacted]
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~ "Gratitude is the memory of the heart…" ~

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They are the reason I am here; they are the reason I thrive to be better.

Cell Wall proteome analysis of *Candida albicans* Dse1 mutant

Racha M. Zohbi

Abstract

The diploid fungus *Candida albicans* is a common opportunistic pathogen that normally colonizes human mucosal surfaces but can cause a wide variety of diseases in an immunocompromised host. *C. albicans* infections, known as candidiasis, range from mild superficial to severe systemic candidiasis in case of *C. albicans* dissemination in the blood stream. In a pathogen, the cell wall and cell wall proteins are important virulence factors and antigenic determinants since they are the first elements to contact the host. Thus, an in depth investigation of the cell wall structure may help reveal novel characteristics behind Candida's virulence. Dse1 is a cell wall protein that has been previously characterized in our lab by homologous recombination of marker cassettes creating a heterozygous stain. The strain was found to be attenuated in virulence, less resistant to cell surface disrupting agents such as calcofluour white, delayed in adhesion to human epithelial cells and deficient in biofilm formation. The current study aims to investigate the cell surface proteome to determine differences in protein expression patterns that might explain the above-mentioned phenotypes. As such the amount of total cell wall proteins in the mutant was found to be lower than in the wild type under filamentous conditions. Furthermore chitin content in the mutant was found to be reduced by 16%, possibly explaining the decreased resistance to calcofluour white, a cell wall disrupting agent that interferes with chitin microfibril assembly. Extracted proteins were then digested with trypsin and analyzed using MALDI-TOF MS, generating a mass spectrometric profile for each strain, each under different growth conditions. These different profiles were compared, and unique peaks for each strain were entered into MS-Fit search engine, compared against a Candida database, and identified by peptide mass fingerprinting (PMF). As such the mutant was shown to lack the chitin biosynthesis protein CHS5, possibly explaining the decrease in chitin biosynthesis. PMF analysis also suggested a mutant-specific expression of glucoamylase 1, a cell wall glycoprotein involved in carbohydrate metabolism and cell wall degradation, changing the cell wall organization and decreasing biofilm formation, and a decrease in lipase protein expression in the mutant, resulting in reduced virulence.

Keywords. *C. albicans* , Dse1, cell wall proteins, chitin, MALDI-TOF MS.

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LIST OF ABBREVIATIONS

WT: Wild type

MALDI-TOF: Matrix assisted laser desorption ionization- time of flight

MS: Mass spectrometry

LC: Liquid chromatography

GPI: Glycosyl-phosphatidylinositol

aa: amino acids

PMF: Peptide mass fingerprinting

PDA : Potato dextrose agar

PDB : Potato dextrose broth

FBS : Feotal bovine serum

DTT : Dithreiothreitol

IAA : Iodoacetamide

β-ME : β-Mercapto-ethanol

BCA: Bicinchoninic acid

Chapter One

Introduction: Backgroud and Significance

1.1. Candida and Candidiasis – Facts and Statistics

Candida albicans is commensal, opportunistic fungal pathogen that can threaten the human population with its intermittent pathogenenicity. Infections caused by *C. albicans* are known as candidiasis and they can affect the skin, the oral cavity and the esophagus, the gastrointestinal tract, the vagina, as well as the vascular system through which *Candida* enters the bloodstream and causes life-threatening systemic infections (Pfaller et Diekema 2007).

Since *Candida* is essentially commensal, viable organisms can normally exist -for example- in the oral or the vaginal cavities of an individual (Cannon et Chaffin 1999 ; Fidel 2007) but once a microbial imbalance or a transient immunosuppression takes place, *C. albicans* can readily proliferate and cause oral candidiasis (thrush) or vaginal candidiasis (Ellepola et Samaranayake 2000). The dissemination of *C. albicans* into the bloodstream usually happens in immunocompromised patients (Fidel 2005) or following surgeries where contaminated medical devices harboring *C. albicans* biofilms are introduced into the patient's body (Chandra *et al*. 2001).

These, coupled with the lack of an early and accurate diagnostic procedure, the high toxicity exhibited by the most common and effective treatments, and the emergence of resistant strains due to empirical prophylactic treatment result in a very high morbidity and mortality rates associated with disseminated infections. (Cozad et Jones 2003).

Internationally, *Candida* is the fourth most common cause of nosocomial bloodstream

infection and invasive candidiasis has a mortality rate of 40-50%, with high costs of hospitalization (up to $40,000 per patient). In the U.S. alone, 50% of total mortalities are related to disseminated candidiasis with billions of dollars of treatment costs per year (Viudes et al., 2002).

Accordingly, the investigation of candidiasis epidemiological burden as well as the host-pathogen interaction in *C. albicans* is important for the development of preventive strategies and new or alternative therapies for an efficient management of patients suffering from invasive candidiasis (Ruan *et al.* 2009).

1.2. Virulence and Pathogenicity

The pathogenicity of *C. albicans* and the disease progression depend on the microorganism itself, as well as the host immune system.

1.2.1. Dimorphism

C. *albicans* is a dimorphic fungus: it grows either by budding (Figure 1-a) or by production of germ tubes (forming hyphal filaments) (Figure 1-b) and can also switch from the budding yeast cell form to the filamentous growth form (hypha). Although both morphologies are found simultaneously in infected tissue, formation of mycelial filaments is thought to play an important role in pathogenesis (Martchenko et al. 2004). This dimorphic transition is essential for virulence and the ability of C. *albicans* to invade host tissues is largely dependent on this morphogenetic conversion since filaments aid in the penetration of tissue required for dissemination while yeast cells are necessary for clonal expansion. This morphology switching is essential; strains that are locked in either form are avirulent. This transition is basically regulated by

temperature, serum, extracellular pH (physiological pH 7), CO_2 concentration, hypoxia, etc. (Karkowska et al. 2009).

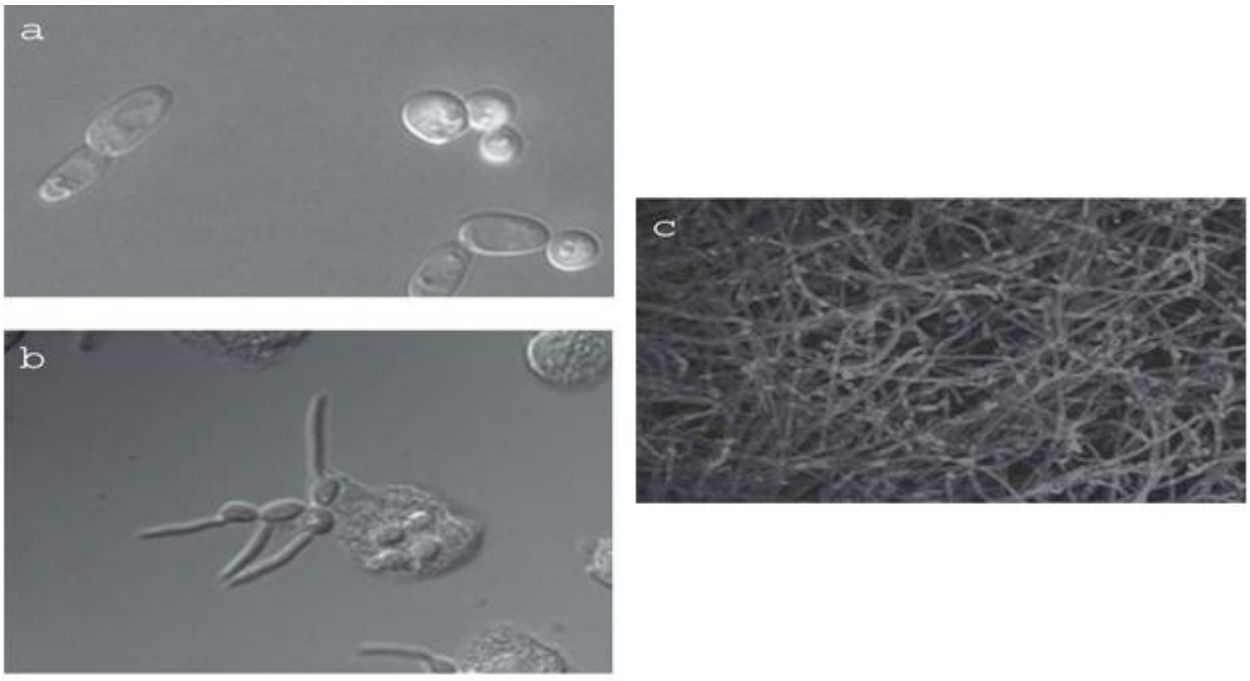

Figure 1: **Various morphological forms of *C. albicans*.**
(**a**) Budding yeast morphology (**b**) Hyphal phase with elongated germ tubes; shown here with an engulfing macrophage (**c**) Biofilm *(Magee 2010)*

1.2.2. Virulence Factors

Complementing the yeast-hyphae transition and switching, efficient virulence factors secreted by *C.albicans* aid in host tissue colonization and the maintenance of cell wall integrity, causing disease, and overcoming host defenses (Naglik et al. 2003). Virulence factors include hydrolytic enzymes that have two major contributions: the provision of nutrients (through the digestion of the extracellular matrix) and to support fungal penetration of host barriers (host tissue destruction) (Kretschmar et al. 2002).

The three most significant extracellular hydrolytic enzymes produced by *C. albicans* are the secreted aspartyl proteinases (the Sap proteins, encoded by a family of 10 *SAP* genes), phospholipases (most imposrtantly phospholipase B enzymes (PLB), lipases (LIP), and adhesins such as the Als family and Hwp1 (Sundstrom 2002).

Sap production is coupled to other putative virulence attributes of *C. albicans* including hyphal formation, adhesion, and phenotypic switching and it aids in the spread of *C. albicans* and the development of localized and disseminated infections (Felk et al. 2000).

SAPs break down laminin, fibronectin, cystatin A, salivary lactoferrin, complement proteins and other components of host tissue (Naglik et al. 2003).

SAP genes may be regulated through the proteolytic activity of other family members (Naglik et al. 2003), and are expressed in a stage-specific and niche-specific fashion. Each protease expresses optimal function at a specific pH, with the family covering a wide range of activity from low pH (*SAP3*) to high pH (*SAP6*). Sap enzymes also acquire different substrates: SAP1, SAP3, SAP4, SAP7 and SAP8 are upregulated during oral disease, while SAP1, SAP3 and SAP6-8 are linked to vaginal infection (Taylor et al. 2005).

No less than ten members of the LIP family are identified (LIP1-LIP10). Their lipolytic activity enables *C. albicans* to grow on lipids as the sole source of carbon, though they are expressed in a flexible, lipid-independent manner (LIP2 and LIP9 are only expressed in the absence of lipids; LIP3-LIP6 are expressed in all media). Most lipase genes are also expressed during the yeast-to-hyphal transition and during infections, contributing to the persistence and virulence of *C. albicans* in human tissue (Hube et al. 2000).

Phospholipases (PL) include *PLA1* and *PLA2* that cleave the ester bonds in glycerol molecules, *PLC* and *PLD* that hydrolyze amphipathic phospholipid molecules (Theiss et al., 2006), and -the major phospholipase in *C. albicans*- phospholipase B.
Phospholipase B (PLB) is secreted at elevated levels during infection, and it cleaves the ester bonds in glycerophospholipids causing membrane distruption and dysfunction. The fungus can then easily cross host cell membranes, and the infection is rapidly disseminated (Ghannoum 2000).

Als (agglutinin-like sequence) proteins and Hwp1 promote adhesion of fungal cells to host tissue (Sundstrom 2002). ALS gene family encodes eight cell-surface GPI-anchored proteins that support the adhesion of *C. albicans* cells to host tissue and that are also differentially regulated in a niche-specific fashion (Hoyer 2001). HWP1 encodes a cell-surface adhesin that confer strong interactions between C. albicans and host cells and that is highly expressed during hyphal development (Klim et al. 2007).

Furthermore, *C. albicans* can avoid or overcome the host immune system by releasing diverse catalases, dismutases, heat shock proteins and other virulence factors that help protecting the pathogen from Reactive Oxygen Species (ROS) burst generated by the host macrophages (Brown et al., 2007).

1.2.3. Biofilm Formation

Candida can colonize surfaces and medical equipments causing nosocomial and persistent infections (Pappas et al. 2004). Biofilm is composed of cells attached to a surface and embedded in a matrix produced by the organisms (Donlan 2001). The film contains both hyphal and budding cells (Figure 1-c). Biofilm formation is accompanied by phenotypic changes like the resistance to antifungal agents which render the treatment of biofilm infections more challenging (Al-Fattani et al. 2004).

1.2.4. Stress adaptation

Stress adaptation is also essential for the virulence of *C. albicans*. Stress adaptaion genes, like catalase, superoxide dismutase and components of the glutaredoxin and thioredoxin systems, are usually induced when *C. albicans* cells are exposed to macrophages, neutrophils, blood or epithelial cells. This exposition also induces the heat shock proteins (chaperones) that provide protective functions (Enjalbert et al. 2003).

Thus, dimorphism and switching, virulence factors and environmental adaptation are all essential and required for the pathogenicity of *C. albicans.*

1.3. Cell wall

The cell wall is an extremely important cellular structure in *C. albicans*. It harbors the essential proteins that control *Candida*-host adhesion; stress tolerance, morphogenesis, immunogenesis, as well as the sites for possible therapeutic and diagnostic markers (Masuoka, 2004). Therefore, the structure of the cell wall is crucial for *C. albicans* virulence and its importance resides in its composition.

The cell wall is composed up to 80 to 90% of carbohydrates. The major polysaccharides of the cell wall are represented by three basic constituents:

- branched polymers of glucose containing β-1,3 (a stress tolerant glucan) and β-1,6 (water soluble glucan) linkages (β -glucans);
- unbranched polymers of *N*-acetyl-D-glucosamine (GlcNAc) containing β-1,4 bonds (chitin, a polysaccharide that tolerates stress);
- polymers of mannose (mannan) covalently associated with proteins (glycomanno proteins).

In addition, cell walls contain proteins (6 to 25%) organized in a bilayer form and minor amounts of lipid (1 to 7%) (Ten cate *et al*., 2008).

These cell wall components are structured as follows: the most internal layer is composed of mannoproteins, linked to chitin at their proximal regions (i.e. together, they make up the periplasmic space). Outwards of the mannoproteins is a layer that is composed of a β-1,3-glucan scaffold, linked to a β-1,6-glucan scaffold: the external component of the two glucan layers to which some cell wall protein and chitin are usually linked (Tronchin *et al*., 1981).

Based on their location of attachment to the glucan layers, the cell wall proteins are divided into two categories : the first are linked to the β-1,6-glucan layers through a glycosylphosophatidylinositol (GPI) remnant and these proteins are called GPI anchored proteins. (Figure 2). Other proteins, such as the Pir (Proteins with Internal Repeats) family are directly linked by covalent bonds to the β-1,3-glucan layer (Kapteyn *et al* 2000).

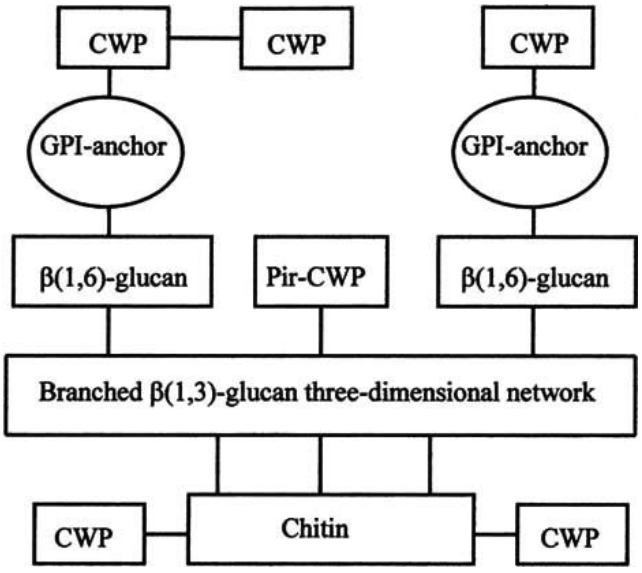

Fig 2. Molecular organization of *C. albicans* cell wall. A scaffold of branched β(1,3)-glucan serves for the covalent attachment of other cell wall components; covalent linkages (as disulfide bridges) between different cell wall mannoproteins (CWP) also occur, and some mannoproteins are linked to chitin. *(Gozalbo et al. 2004)*

As previously mentioned, mannose polymers (mannan) are found in covalent association with proteins (mannoproteins) and represent about 40% of the total cell wall polysaccharide. The term “mannan” has been used also to refer to the main

soluble immunodominant component present in the outer cell wall layer of *C. albicans*, called phosphomannoprotein or phosphopeptidomannan complex. This cell wall fraction contains homopolymers of D-mannose (as the main component), 3 to 5% protein, and 1 to 2% phosphate. The proximal regions of mannoproteins are linked to chitin and, together, they form the peri-plasmic space (Figure 3) (Yadegari et al. 2002).

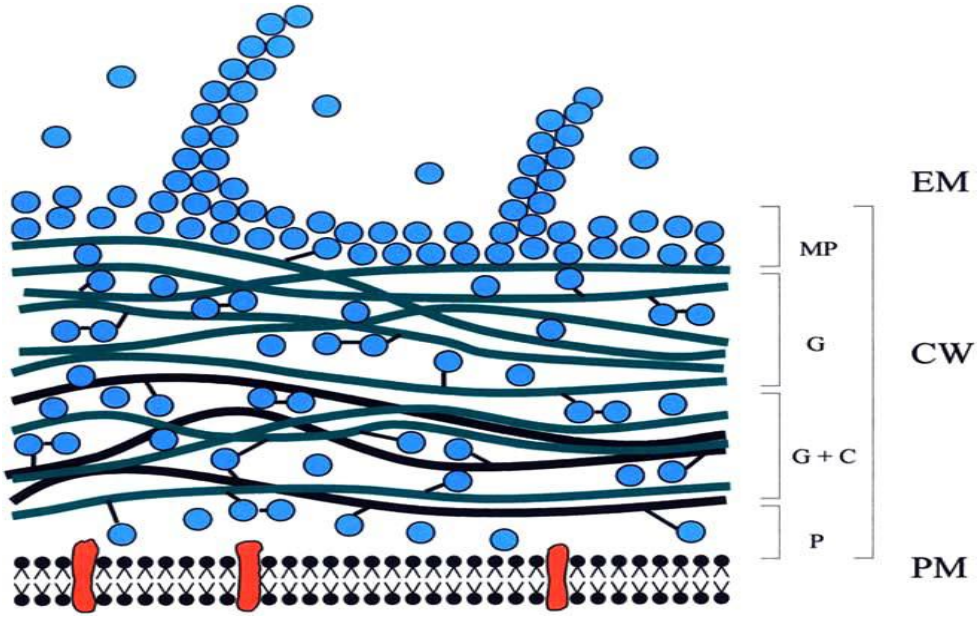

Fig 3 . Schematic diagram of the *C. albicans* cell wall structure. Microfibrillar polysaccharides, glucans (green line) and chitin (black line) are covalently linked and constitute the skeletal network of the cell wall (CW). Mannoproteins (blue circles) expand the cell wall, from periplasm (P) to the external surface, and some may be secreted to the extracellular medium (EM). Different covalent linkages (sticks) formed between some mannoproteins and polysaccharide chains and between different mannoproteins, contribute to cell wall organization. MP=mannoproteins. G=Glucans. C=Chitin. P=Periplasm *(Gozalbo et al. 2004)*

Mannoproteins play a central role in the course of *C.albicans* adherence to mucosal surfaces, allowing the organism to cause infection. Several studies suggested that mannoproteins are the main antigenic cell wall components and they can consequently be used as basic antigen during antibody development. Hence, the

investigation of the cell wall proteins – and specifically the mannoproteins- of *C. albicans* is of immense importance in order to understand the actual mechanism of infection leading to probable prevention or treatment of the disease (Sandini et al. 2007).

Chitin is a β-1,4-homopolymer of N-acetyl glucosamine. In *C. albicans* four chitin synthase enzymes exist:

- CaChs3p (mostly regulated at the post-transcriptional level),
- CaChs2p, CaChs1p and CaChs8p (can be transcriptionally activated due to the stimulation of some specific signaling pathways, such as the PKC, Ca^{2+}-calcineurin and HOG pathways).

Chitin, together with β-1,3-glucan (being the major structural polysaccharides in fungal cell walls) counteract cell turgidity, establish cell shape and attribute to structural rigidity.

Several studies revealed chitin synthesis as a critical feature of fungal growth that is also crucial for fungal cell viability: any reduction in glucan levels in the cell walls, following induced mutations in glucan synthase genes, stimulate a "recovery pathway" in which chitin synthesis is enhanced, restoring the strength of the cell wall (Popolo et al. 2001).

In addition, chitin synthesis was found significant in fungal morphogenesis: Hyphal cell walls have a greater chitin content than yeast cell walls and the specific activity of chitin synthase is two-fold higher in hyphae than in yeast cells (Munro et Gow, 2001)

1.4. Protein Identification using Mass Spectrometry

The proteome represents the sum of proteins expressed or encoded by a genome. Each protein being composed of amino acid subunits, protein can be analytically recognized my mass spectrometry through their digestion into peptides, then weighing

the unique mass of their individual amino acid composition. The resulting recognition of the amino acid sequence can help identifying the protein of which they encompass.

Mass spectrometers are mainly divided into three essential parts: the ionization source, the mass analyzer, and the detector. Samples are first exposed to an ionization source where the sample molecules are ionized. Ions are then extracted into the analyzer region, where they are separated based on their mass-to charge (*m/z*) ratios. Separated ions are detected and the signal is sent to a computer system where the *m/z* ratios and their relative abundance are stored and presentated as spectrum of intensity versus *m/z* (Baldwin, 2005).

MALDI-TOF: Matrix assisted laser desorption ionization (MALDI) can analyse thermolabile, non-volatile organic compounds, particularly those of high molecular mass.

For this technique, the isolated sample (protein samples in our study) is digested with an enzyme with known cleavage specificity (usually trypsin), then pre-mixed with a highly absorbing matrix compound such as 3,5-dimethoxy-4-hydroxycinnamic acid (sinapinic acid), α-cyano-4 hydroxycinnamic acid (alpha-cyano or alphamatrix) (CHCA) or 2,5-dihydroxybenzoic acid (DHB) (Figure 4).

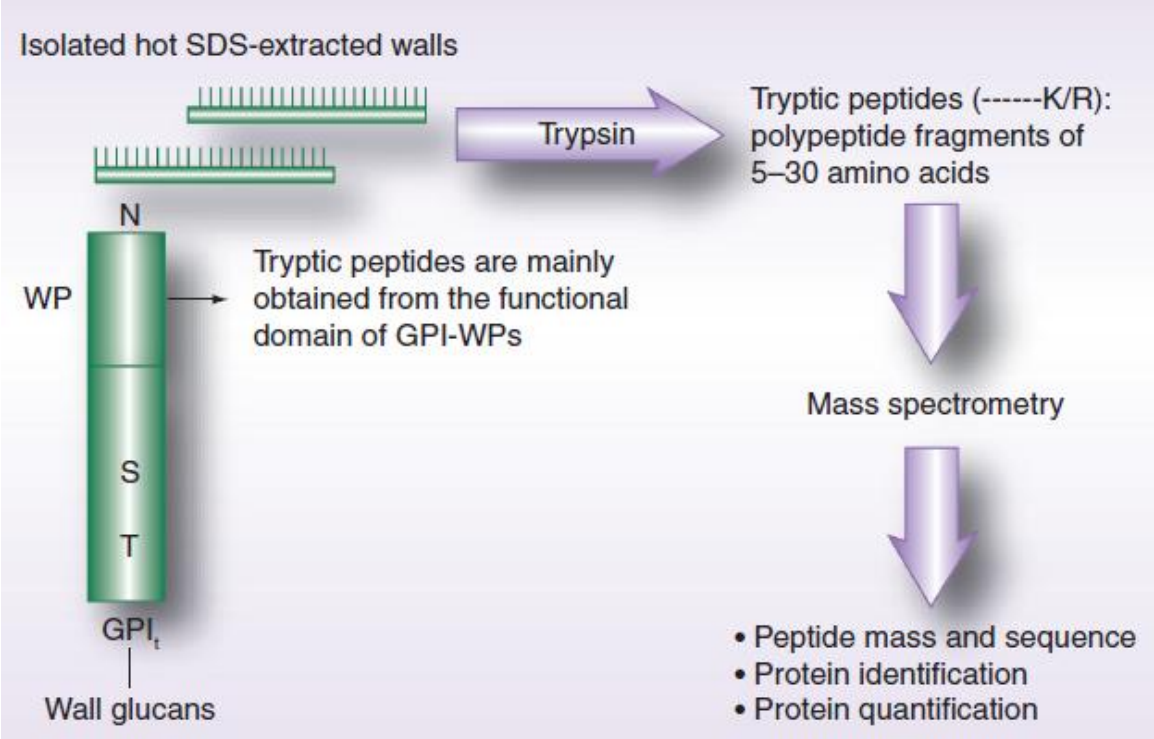

Fig.4- Mass spectrometric analysis of wall proteins. Fragmented walls are first extracted with hot SDS to release noncovalently bound proteins and then treated with trypsin. Most tryptic peptides originate from the N-terminal part of the WPs. The tryptic digest is subsequently analyzed by mass spectrometry. GPI:Glycosylphosphatidylinositol; GPIt: Truncated GPI-anchor that interconnects a GPI-WP with the β-glucan network; SDS: Sodium dodecyl sulfate; WP: Wall protein. *(Klis et al. 2011)*

MALDI ion source is generally coupled with TOF mass analyzers (MALDI-TOF). Computational algorithms such as MASCOT (www.matrixscience.com) or MS-FIT (http://prospector.ucsf.edu) coupled to MALDI-TOF permit the categorization of proteins according to their peptide mass fingerprint.

Peptide mass fingerprinting (PMF) identifies protein by comparing the experimental mass spectrometric profiles of enzymatically digested peptides, to theoretical profiles of *in silico* digested peptides stored in databases. According to a survey published in 2007 by Damodaran et al., 68% of proteomic studies utilize PMF for protein identification, revealing the great value of PMF in proteomics.

Another method of protein identification is the MS/MS method. It consists of the additional fragmentation of each digested peptide into smaller sequences, ultimately providing effective signatures of individual amino acids in each peptide. MS/MS method may be more specific for peptides recognition, but it is also much more expensive and time-consuming than PMF (Song et al. 2010).

Microorganisms (such as *C. albicans*) have a relatively small genome that can easily be genetically manipulated. The consequential variation in protein expression and the related functions of the corresponding proteins can be studied through applying proteomic research. This would help understanding the host-pathogen relationship, tracking the pathogen response to the different therapies and exposing probable novel sights for clinical intervention (Evangelou et al. 2007).

In *C. albicans*, cell wall proteins are of great interest because of their direct contact and potential interaction with the host. These proteins may act as adhesins, elicit an immune response and vary with morphological and environmental condition (Chaffin et al. 2008).

Mass spectrometric analysis has been applied to investigate *C. albicans* secreted proteins (secretome) as well as cell wall proteins. MALDI-TOF MS analysis of intact yeast cells was used to identify yeast species (including *C.albicans*), and compare the different mass-to-charge signatures for the classification of fungal cells at the genus and species levels (Qian et al. 2008).

A study by Cabezo´n et al. in 2009 claimed the identification of nearly 214 membrane proteins using MALDI-TOF technique. They included 41 plasma membrane proteins, 20 plasma membrane associated proteins, and 22 proteins with unknown membrane localization, and 12 GPI-anchored membrane proteins. The detected proteins appeared to be involved in biopolymer biosynthesis, transport processes, cell wall β-glucan synthesis and maintenance, and virulence.

Mass spectrometric analysis of the secretome of *C. albicans* grown under different growth conditions, led to the identification of 44 secretory proteins, a soluble form of the transmembrane protein Msb2, six proteins predicted to be associated with compartments of the secretory pathway and 28 cytosolic. The same study proved that many covalently anchored wall proteins are partially released into the growth medium and that the protein composition of the secretome changes considerably in response to environmental conditions. (Sorgo et al. 2010)

Mass spectrometry was also used to identify the differentially expressed proteins in the closely related organisms *C. albicans* and *C. glabrata,* enlightening the mechanisms responsible for distinct biological features of clinical importance (Prasad et al. 2010).

1.5. DSE1 gene

Daughter Specific Expression gene 1 (Dse1) is a *C. albicans* cell wall protein involved in cell wall metabolism and required for the separation of the mother and daughter cells. Dse1 usually appears to be periodically expressed, with a peak expression at the M/G1 phase (Cote et al. 2009).

The generation of a *dse1* homozygote null strain was so far unachievable: one copy of the Dse1 gene is always conserved in all transformants with two Dse1 alleles knocked out, although different techniques and cassettes were tried (Daher et. Al 2011). This can imply that Dse1 is an essential gene in *C. albicans* **.**

A mutant haploinsufficient strain (lacking one copy of the gene) was generated in our lab using DNA cassettes containing the functional *URA3* or *HIS1* markers, flanked by 100 bp of *Dse1* 5' and 3' regions. Homologous recombination and integration created a *DSE1/URA3::dse1* and a *DSE1/HIS1::dse1* heterozygous strain.

The mutant strain showed a reduced resistance to calcofluor white (a cell wall disrupting agent) that targets chitin microfibril assembly thus weakening the cell wall. The mutant also showed a decreased resistance to SDS (a detergent disrupting and

solubilizing the plasma membrane), that was explained by an increased permeability to SDS due to the cell wall weakening in the mutant strain.

Additionally, the mutant sensitivity to hydrogen peroxide increased as well, suggesting that the decreased amount of Dse1 protein on the cell surface altered the architecture of the cell wall proteome, preventing the correct positioning of the proteins responsible for countering oxidative stress damage.

Moreover, the mutant strain expressed a reduced ability to form biofilm although it exhibits a delayed -but existent- adhesion. The causes behind the delay in biofilm formation require further investigations. This defect in biofilm formation and the delay in adhesion may have lead to the observed reduction in the virulence of the mutant strain. Finally, the Dse1 heterozygous strain presented a hyperfilamentous phenotype that might be due an upregulation of a filamentation pathway to compensate for the Dse1 deletion (Daher et al. 2011).

1.6. Aim of the study

As previously discussed, the heterozygote Dse1 mutant strain exhibited certain phenotype discrepancies when compared to the parental wild type strain.

The reduced resistance of the mutant strain to cell wall disrupting agents might be attributed to a decreased chitin synthesis resulting in weakening of the cell wall. Additionally, and in light of the importance attributed to cell wall proteins in virulence and host/pathogen interaction, any differential expression of cell wall proteins can also explain the mutant's decreased resistance to oxidative stress, delayed adhesion, reduced ability to form biofilm and ultimately reduced in virulence.

Therefore our project aims to assess the total chitin content and total cell wall protein content of the filamentous and non filamentous form of the mutant strain in comparison to both forms of the parental strain. Moreover, the project involves the

determination of mutation-specific cell wall proteomic profiles by mass spectrometric analysis using MALDI-TOF of total cell wall proteins, of the mutant and the parental wild type strains, under both filamentous and non filamentous growth conditions, followed by the identification of differentially expressed proteins through PMF analysis. Any differences observed would be key in explaining the observed mutant phenotype.

Chapter Two

Research Design and Methods

2.1. Strains and culture conditions

Both mutant and parental strains were previously generated in our lab (Daher et al. 2011).

For filamentous conditions, both strains were cultured without selection on rich potato dextrose agar (PDA) and incubated at 37 °C overnight. Colonies were then selected and grown in dextrose broth (PDB) with 20% Fetal Bovine Serum (FBS; BioWhittaker, Walkersville, MD, USA) and incubated at 37 °C overnight. Cells were then harvested by centrifugation, washed with cold distilled water and then resuspended in 50 mM Tris buffer.

For non filamentous conditions, all strains were cultured without selection on rich PDA medium (Himedia Laboratories, Mumbai, India) and incubated at 30 °C overnight. Colonies were subsequently selected and grown in potato PDB incubated at 30 °C overnight. Afterwards, cells were harvested by centrifugation and washed with cold, distilled water and resuspended in 50 mM Tris buffer.

2.2. Cell wall extraction and isolation

Cell wall extraction and isolation were completed following a modified protocol. (Clemens, J.H., Sorgo, A.G., Siliakus, A.R., Dekker, H.L., et al. 2011). Cells were spun at 4000 rpm for 5 min at 4 °C. The pellets were subsequently washed three successive times with cold distilled water and then resuspended in 5 mL 50 mM Tris, spun again and resuspended in 1 mL of the same buffer. Samples were then mixed with 2 µL of protease inhibitor cocktail (SIGMA-ALDRICH, P8215) and glass beads of 0.5 mm diameter and subjected to 30 rounds of vortexing

where each round included 30 sec vortexing and 30 sec on ice. An orange color starts to appear, representing a reaction between the protease inhibitor and the acidic cytosol. The expected cell breakage was checked under microscope. Samples were next kept on ice for 5 min and supernatants were then transferred to new clean tubes. The remaining beads were washed three times with 1 M NaCl and the resulting supernatants were also transferred to the new tubes.

Cell lysate was then centrifuged for 5 min at 3000 rpm at 4 °C and supernatant was carefully discarded. Pellet was washed until supernatant became clear with 1 M NaCl and cold distilled water. Samples were resuspended in a small volume of distilled water and transferred to pre-weighed 12 mL tubes. Tubes were then centrifuged for 5 min at 3000 rpm and supernatant discarded and pellet weighed.

SDS extraction buffer (50 mM Tris, 2% SDS,100 mM Na-EDTA, 150 mM NaCl, pH= 7.8, 8 µL/1 mL β-ME) was added to samples as 0.5 mL of buffer per 100 mg wet weight and resuspended completely. Tubes were then boiled for 10 min and left to cool to room temperature. Then tubes were spun at 3000 rpm for 5 min and supernatant discarded. SDS extraction buffer was added again to the samples which were then subjected to 4 cycles of boiling for 10 min, cooling to room temperature and then spun for 5 min at 3000 rpm and supernatant was discarded. Pellets were washed extensively with distilled water until foam disappeared and complete removal of SDS. Pellets were then stored at -20 °C until further analysis.

2.3. Total cell wall proteins quantification

Total cell wall protein determination was done following a standard BCA Assay following manufacturer's protocol (SIGMA- ALDRICH, BCA1-1KT).

At first, 50 mg of wet weight cells were transferred from frozen pellets to a 1.5 mL eppendorf tube and resuspended in 100 µL of 1 M NaOH, boiled for 10 min in a

heating block, cooled to room temperature and 100 µL of 1 M HCl was added. Samples were spun at 13,000 rpm for 5 min and supernatant was transferred to a new tube. Pellet was conserved for chitin assay.

A further 10 µL of the supernatant was then added to 1 mL BCA mix [SIGMA-ALDRICH, BCA1-1KT], (A: B=50:1) and incubated for 30 min at 37 °C. Absorbance was measured at 563 nm and calibration was done with a BSA standard curve ranging from 0-1000 µg/mL protein concentrations. Measurements were done in triplicates and standard error calculated.

2.4. Total chitin content quantification

Total chitin content determination was done following a modified protocol described previously (Munro.et al. 2003, Kapteyn et al. 2000). Briefly, 50 mg of wet weight cell wall pellet was hydrolyzed in 1 mL of 6 M HCl and samples were incubated at 100 °C for 17 hours in a heating block. Samples were then centrifuged at 13000 rpm for 12 min and the supernatant discarded. After centrifugation, pellets were reconstituted in 1 mL distilled water and 0.1 mL of the sample were added to 0.1 mL of solution A (1.5 N Na_2CO_3 in 4% acetylacetone) and incubated at 100 °C for 20 min. Samples were then cooled to room temperature and 0.7 mL of 96% EtOH was added. To the mix, 0.1 mL of solution B (1.6 g of p-dimethylaminobenzaldehyde in 30 mL of concentrated HCl and 30 mL of 96% EtOH) and incubated for 1 hour at room temperature. Absorbance of samples was measured spectrophotometrically at 520 nm and values compared to a standard curve of 0-100 µg glucosamine taken through the same reactions. Measurements were done in triplicates and standard error calculated (Younes et al. 2010).

2.5. Reduction and tryptic digestion of proteins

To start with, 50 mg wet weight of previously isolated cell walls were transferred into a 1.5 mL eppendorf tube. A total volume of 101 µL of reducing solution of (10 mM DTT, 100 mM NH_4HCO_3) was added and samples were incubated at 55 °C for one hour. Samples were allowed to cool at room temperature and then spun down and supernatant was discarded. 106 µL of a quenching solution, 55 mM DTT, 100 mM NH_4HCO_3, were added and samples were incubated for 5 min at room temperature. Then, samples were spun down and five washing cycles were performed with 50 mM NH_4HCO_3 to remove DTT and IAA. Cell wall pellet was then resuspended in 160 µL of 50 mM NH_4HCO_3 and 2 µg of trypsin (2 µL of a 1 µg/µL stock) were added and the mix incubated at 37 °C for 16 hours while shaking. Afterwards, samples were vortexed and centrifuged and the supernatant stored at -20 °C for mass spectrometric analysis.

2.6. Mass Spectrometric analyses by MALDI-TOF

For mass spectrometric analysis samples were first desalted using Omix ZipTip C18 10 µL column activated with 50% acetonitrile (ACN) solution, equilibrated with 0.1% TFA solution and eluted with a 0.1% TFA, 50% ACN following manufacturer protocol. A 4700 Applied Biosystems Matrix Assisted Laser Desorption Ionization-Time of Flight instrument was used and calibrated with 4700 CAL mix. 0.6 µL of samples, diluted 1:1 with matrix, were spotted on a 384 well MALDI-TOF plate using 5 mg/mL CHCA matrix following the dried droplet spotting technique (Moskovets, Chen, Pashkova, Rejtar, Andreev, & Karger, 2003). Internal calibration was done with CAL/sample overlayer spots and error was calculated at 11 ppm. Data acquisition and processing were done under reflector mode with a laser intensity of 6670 Hz and 400 hits per sample. A S/N ratio of 30 was set as threshold and spectra were processed by noise filtering and background noise removal. Peaks were deisotoped. Samples were

run in 10 replicas each on a plate, quintuplicate plates were run and respective peak lists were created for all 50 samples. Respective examples of spectra are shown in Appendix.

A software developed by Computer engineer Mazen Naamani allowed generation of a cumulative peak list resulting from N=50 replicate spots with the % of occurrence of each peak considering a 0.001% (11 ppm) error range. These lists were then processed via a second program developed by Computer engineer Imad Koussa. All the peaks corresponding to the matrix, keratin, and autolysis products of trypsin, were excluded from all sample peak lists.

The same program was used again with the wild type peak list as a reference to exclude all WT related peaks from the sample peak lists. Resulting sample peak lists of which both matrix and WT peaks were excluded were then processed using a third program written by Computer engineer Imad Koussa. This program allowed selection for peaks only found in either the mutant only or WT only, in their filamentous or non filamentous form, with an occurrence greater or equal to 50% averaging all filtered entries falling within the 0.001% error range. Resulting peak lists show filamentous and non filamentous mutant-only peaks, and filamentous and non filamentous WT-only peaks after a 1-to-1 comparison of each mutant with the WT under filamentous and non filamentous growth conditions as described earlier.

2.7. Statistical analysis

Data from both total cell wall proteins and chitin content assays were collected and entered into a Microsoft Excel 2007 sheet. T-student statistical test (TTEST) was carried and p-values less than 5% were considered significant. All experiments were performed in triplicates.

2.8. Protein identification

Centroid peptide masses were used to search for protein identification by using MS-fit software (Protein prospector, University of California, San Francisco, http://prospector.ucsf.edu). Database searches were performed against UniProtKB/Swiss-Prot database (a collaboration between the European Bioinformatics Institute (EBI), the SIB Swiss Institute of Bioinformatics and the Protein Information Resource (PIR). UniProt is mainly supported by the National Institutes of Health (NIH) and the European Commission. http://www.uniprot.org/).

Searches were restricted to Candida entries. One of two possible missed cleavages for trypsin digestion were allowed. Oxidation of Met was considered as a variable amino acid modification.

Chapter Three

Results

3.1. Dse1 – Protein Insight

According to the Uniport Database, C. albicans Dse1 is a 724 a.a. protein (Figure 5), whose molecular weight is nearly 81073.26 Da (Figure 6).

```
  1   MTDYYEPTFLFRQNAIKRFSPSLSPISSVESLSLVDSNSISNNGTTTTTTTTTSSIYQNASNSSWLLNPYNVKDTAILNQ
 81   SCCLNRTNVKSNYWKIPDESMNLTSMSINKNQGGNPILAISSGKSESNLFIYELNLFNNHLIHHHTISLPNIHAMKWINN
161   TRYLVTGNNKGYAHLVSTPKLATHDVFNTNSNGDFDYNDDDEDDNNSAEICKRFNHRKHLKQNQLENTISTPIKHLNFLN
241   NHENLLSIYNDYLFYWDIKGCHQQTRPSPISISTVSGIKNFDVPEKNHSSTNTSSANTVAICGLFGVSLFDLRDCQFNIP
321   NYNTAQIHDKTQSSYRKLSANIVKWNPMNTNILAAGHGDGVIRLWDIRKQDSYIAELYGHNNYSITTSMEWNNNDLFTGS
401   KDGNIIHWDLSNISKDSQWEENDNTKLPISCGLKEGFNSIEFNGKANKLETKLDQYQCGTILPASNSSIVAMCSTTTTTS
481   SNDHDDEEIKILSIDSSSFLGVHNKVSESINVNINTNKLYYTEEDIQLLLQSQLNNNNITSSSATGSNDTLISFGNNVSQ
561   DSLVKPLTISRKPTTTIKNNNNNTTTRPTNTHTHSASIDESIVSVHNVSNDTLVNSPTRFNICESEDEFTFNYVKNNTNN
641   EDQRQSDNRNSSFSSVSSQQTQLNNSVESLSTVVTDVENEVNHQEHKYMSLLLPVKDTSTTTTTTKDNNTNSDQAVTNTP
721   IISI
```

Fig.5- *C. albicans* Dse1 amino acid sequence . UniProt Database www.uniprot.org

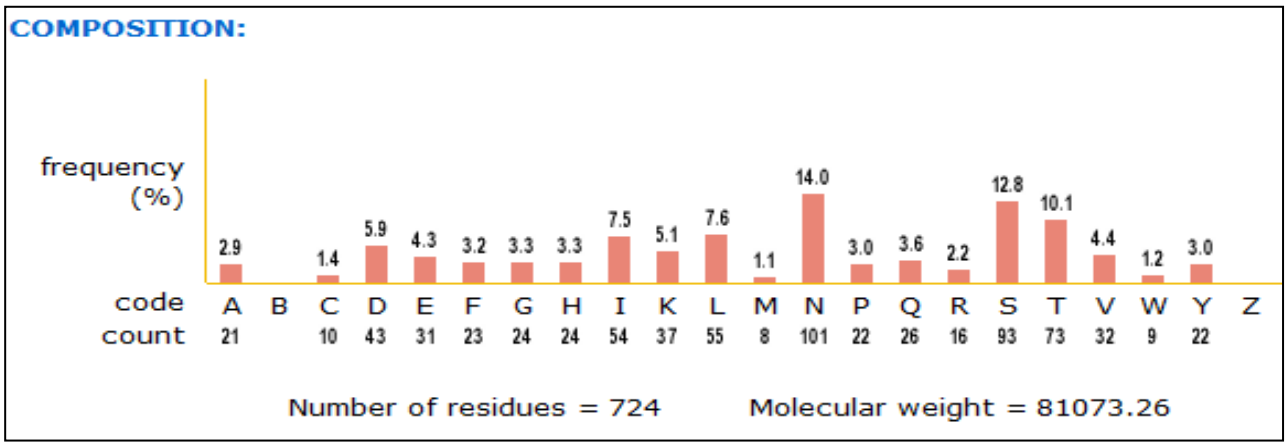

Fig.6- *C. albicans* Dse1 amino acid composition : A21 C10 D43 E31 F23 G24 H24 I54 K37 L55 M8 N101 P22 Q26 R16 S93 T73 V32 W9 Y22. With A= Ala. B= Asx. C= Cys. D=Asp. E= Glu. F=Phe. G=Gly. H=His. I=Ile. K=Lys. L=Leu. M=Met. N=Asn. P=Pro. Q=Gln. R=Arg. S=Ser. T=Thr. V=Val. W= Trp. Y= Tyr. Z= Glx. www.candidagenome.org

Table 1 - *C. albicans* DSE1 *in silico* digest

m/z		
803.9013	1680.9485	
803.9393	1693.9145	2737.1348
831.0714	1696.9478	2744.9774
831.0114	1705.6644	2763.2139
848.9365	1712.9472	2779.2133
869.9584	1865.1529	2813.1377
873.0899	1894.1923	2927.3295
909.0360	1918.0366	2956.1197
1057.1058	1963.2577	2960.1190
1064.3839	1986.1774	3018.4564
1073.2445	2007.2672	3031.4138
1080.3833	2022.2169	3034.4558
1105.0719	2037.3219	3050.4551
1140.2919	2049.3048	3072.2265
1242.3386	2053.3213	3200.6030
1356.5307	2054.3873	3609.6230
1366.3483	2097.4157	3643.0514
1432.5829	2102.4670	3697.9535
1451.6735	2118.4664	3713.9529
1452.6163	2138.4650	3714.9842
1469.6445	2154.4644	3730.9836
1486.6752	2178.3970	3765.8116
1494.6538	2180.5026	3799.3763
1555.6976	2196.5019	3815.3757
1583.8141	2359.6988	3843.1594
1599.8134	2375.6981	3859.1587
1612.7934	2391.6975	
1617.8537	2721.1354	
1625.8516		
1641.8510		

In silico tryptic digestion using Protein prospector yielded DSE1-specific sets of expected m/z values

3.2. Total cell wall protein content

Total cell wall protein content under filamentous and non filamentous conditions

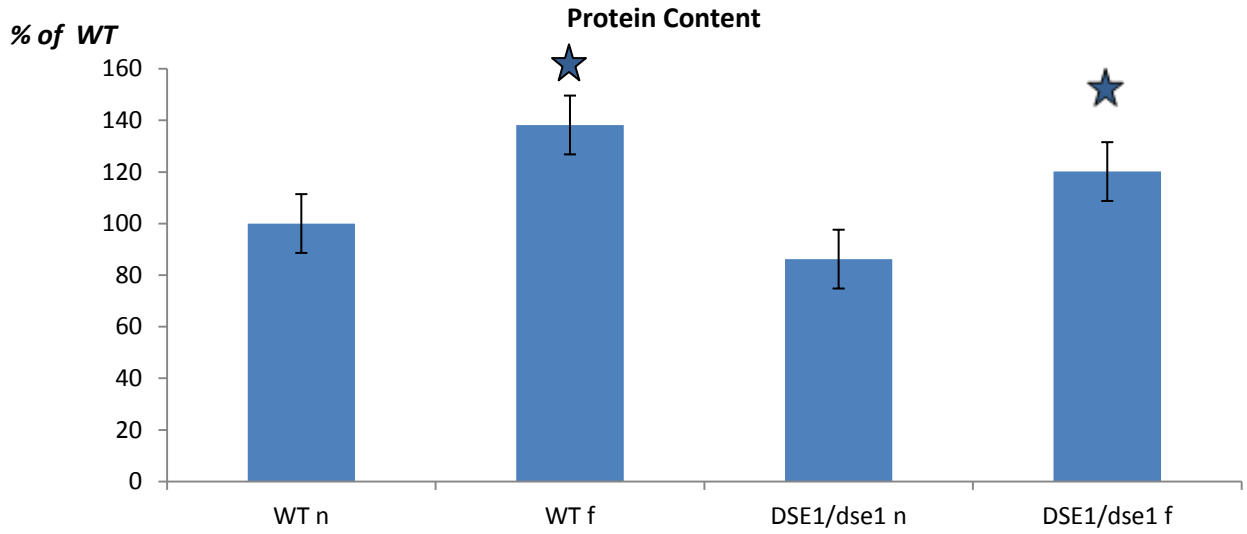

Fig.7- Graph showing total cell wall protein content in the mutant with reference to the wild type strain. Bars displayed represent ±SEM. WT n and DSE/dse1 n strains were grown under non filamentous conditions in PDB at 30 °C. WT f and DSE1/dse1 f strains were grown under filamentous conditions with 20% FBS in PDB at 37 °C. Measurements were done in triplicates. Statistically significant data with p< 0.05 are annotated with a star.

Total cell wall protein content under filamentous and non filamentous conditions was assayed and results are displayed in figure 7. The parental strain grown under filamentous condition showed a 38% increase in total protein content compared to WT, whereas the DSE1/dse1 mutant strain grown under non filamentous conditions showed a 14% decrease in total protein content with respect to the WT and the DSE1/dse1 mutant strain grown under filamentous conditions strain showed a 20% increase in total protein with reference to WT strain.

3.3. Total chitin content

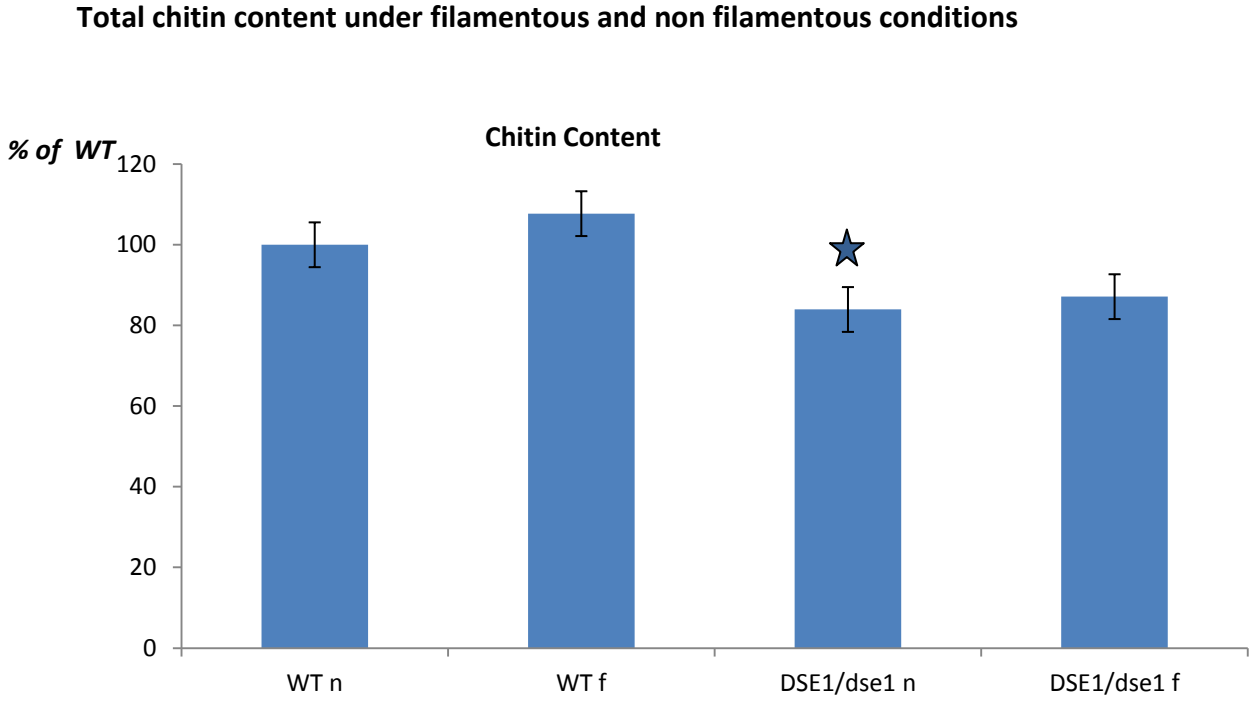

Fig.8- Graph showing total chitin content in the mutant with reference to the wild type strain. Bars displayed represent ±SEM. WT n and DSE1/dse1 n strains were grown under non filamentous conditions in PDB at 30 °C. WT f and DSE1/dse1 f strains were grown under filamentous conditions with 20 % FBS in PDB at 30 °C. Measurements were done in triplicates. Statistically significant data with p< 0.05 are annotated with a star.

Chitin assay was performed on the parental and mutant strains under filamentous and non-filamentous conditions. The total chitin content of the parental strain grown under filamentous conditions only showed a non statistically significant increase of 8% with respect to the parental strain grown under non filamentous conditions.

The *DSE1/dse1* mutant strain grown under non filamentous conditions revealed a 16%

decrease in total chitin content with respect to the WT strain, whereas the mutant strain grown under filamentous conditions showed a 13% decrease in total chitin content compared to the WT strain.

3.4. - MALDI-TOF Mass spectrometric analysis of cell wall proteomes

3.4.1.*DSE1/dse1* cell wall proteins analysis- under non filamentous conditions

The mass spectrometric profile of *DSE1/dse1* heterozygous strain grown under non filamentous conditions displayed around 98% similarity with the wild type strain. The discriminative comparisons between both strains revealed that 2.4% of the peaks were unique to the mutant strain whereas only 2% were unique to the wild type.

Peptide mass fingerprinting (PMF) was used for protein identification. Unique peaks for each strain were compared to Candida theoretical mass spectrometric profiles in UniProtKB/Swiss-Prot databases. The best matches were considered to compute and infer peptide sequences with similar profiles, and to subsequently recognize a theoretical matching protein.

A summary of all the suggested proteins is found in the annex. The tables below show the proteins that are hypothetically relevant to this study.

Table 2- List of proteins that are hypothetically exclusively expressed in the WT strain grown under non filamentous conditions (WT n), not in the mutant strain grown under non filamentous conditions (DSE n)

WT n – DSE n						
Protein Name	**Protein Description**	**Cellular Location**	**Accession number Protein name (CGD)**	**UniprotKB accession number**	**Number of peptide matches**	**% of coverage**

Putative lipase ATG15	Putative lipase	Integral to membrane	CAL0004893. ATG15.	Q5A4N0	4	7.4 %
Putative uncharacterized protein	-	-	CAL0004306. orf19.2797	Q59PU6	7	10.5 %
Chitin biosynthesis protein CHS5	Putative chitin biosynthesis protein	Golgi apparatus	CAL0002612. CHS5	O74161	4	8.5 %
Deoxyhypusine hydroxylase	Biofilm formation	Cytoplasm - Nucleus	CAL0006381. orf19.2286.	Q59Z14	4	7.7 %
Phenylalanyl-tRNA synthetase beta chain	Protein biosynthesis	Cytoplasm	-	O13432	5	9.2 %
5-methyltetrahydro-pteroyltriglutamate-homocysteine methyltransferase	Amino-acid biosynthesis	Cell surface	CAL0002475. MET6.	P82610	4	7.6%
ATP-dependent RNA helicase DED1	Protein biosynthesis	Cytoplasm	CAL0004832. orf19.7392.	Q5A4E2	4	7.4%
Eukaryotic translation initiation factor 3 subunit C	Protein biosynthesis. Response to drug	Cytoplasm	CAL0003672. NIP1.	Q5AML1	6	8.9%
Phosphatidylethanolamine N-methyltransferase	Phospholipid biosynthesis	Membrane	CAL0003197. CHO2.	Q59LV5	7	9.3%
Superoxide dismutase [Cu-Zn] SOD1	Antioxidant Oxidoreductase	Cytoplasm	CAL0006717. SOD1.	O59924	1	1.1 %
pH-regulated antigen PRA1	Glycoprotein. Increase adhesion	Secreted	CAL0066667. PRA1	P87020	2	4.7 %
Mannan polymerase complex subunit MNN9	Protein modification and glycosylation	Golgi membrane	CAL0004800. MNN9.	P53697	4	7.3%

Table 3 - List of proteins that are hypothetically exclusively expressed in the mutant strain grown under non filamentous conditions (DSE n), not in the WT strain grown under non filamentous conditions (WT n)

DSE n – WT n						
Protein Name	**Protein Description**	**Cellular Location**	**Accession number Protein name (CGD)**	**UniprotKB accession number**	**Number of peptide matches**	**% of coverage**
Sorting nexin MVP1	Cell communication, protein transport. Peripheral membrane localization.	Cell membrane	-	Q3MPQ4	6	8.5%
Glucoamylase 1	Glycoprotein. Cell wall biogenesis/degradation- Altered biofilm formation	Cell wall/ Membrane	CAL0066397. GCA1.	O74254	5	7.7%
Autophagy-related protein 17	Autophagy	Membrane/ Cytoplasm.	CAL0006128. orf19.2982.	Q5AI71	4	7.2%
Probable mannosyltransferase MNT2	Possible glycosyltransferase (protein glycosylation)	Cell membrane.	-	P46592	4	8.2%

3.4.2. *DSE1/dse1* cell wall proteins analysis- under filamentous conditions

The *DSE1/dse1* heterozygous strain and the WT strain, both grown under filamentous conditions exhibited around 97% similarity in their mass spectrometric profiles. The discriminative comparisons between both strains revealed that 2.2% of the peaks were unique to the mutant filamentous strain while 2.6% were unique to the wild type filamentous strain.

Candida database search was performed for data analysis and peaks identification. Unique peaks for each strain were compared to Candida theoretical mass spectrometric profiles. The best matches were considered to compute and infer peptide sequences with similar profiles, and to subsequently recognize a theoretical matching protein.

A summary of all the suggested proteins is found in the annex. The tables below show the proteins that are hypothetically relevant to this study.

Table 4- List of proteins that are hypothetically exclusively expressed in the WT strain grown under filamentous conditions (WT f), not in the mutant strain grown under filamentous conditions (DSE f)

WT f - DSE f						
Protein name	**Protein Description**	**Cellular location**	**Accession number Protein name (CGD)**	**UniprotKB accession number**	**Number of peptide matches**	**% of coverage**
SEC14	biofilm-regulated	Cytosol	CAL0003685. SEC14.	P46250	3	6.4%
Eukaryotic translation initiation factor 3 subunit C	Response to drug	Cytoplasm	CAL0003307. PRT1	Q5AGV4	3	7.1%
14-3-3 protein homolog	pathogenesis	Cell surface	CAL0001346. BMH1.	O42766	4	6.9%
GTP-binding RHO-like protein	Lipoprotein-signal transduction	Cell membrane	-	P33153	4	8.3%
Glucosamine--fructose-6-phosphate aminotransferase	Cell wall chitin biosynthetic process	Membrane	CAL0006344. GFA1.	P53704	7	10.2%
Probable NADPH reductase TAH18	Oxidoreductase.	-	CAL0004403. orf19.2040.	Q5AD27	4	8.1%
F-actin-capping protein subunit alpha	Actin cytoskeleton organization	Cytoplasm, Cytoskeleton	CAL0003289. orf19.3235.	Q5A893	4	7%
Pre-mRNA-splicing factor ISY1	mRNA processing-response to drug	Cytoplasm	CAL0004135. orf19.6685.	Q59R35	4	9.2%
ATP-dependent RNA helicase DED1	Protein biosynthesis	Cytoplasm	CAL0004832. orf19.7392.	Q5A4E2	9	14.7%

Table 5- List of proteins that are hypothetically exclusively expressed in the mutant strain grown under filamentous conditions (DSE f), not in the WT strain grown under filamentous conditions (WT f)

DSE f – WT f						
Protein name	**Protein Description**	**Cellular location**	**Accession number Protein name (CGD)**	**UniprotKB accession number**	**Number of peptide matches**	**% of coverage**
Serine/threonine-protein kinase CLA4	virulence and morphological switching	-	-	O14427	9	15.3%
Pre-mRNA-splicing factor CEF1	Cell cycle control	Cytoplasm	CAL0004619. orf19.4799.	Q5APG6	9	14.8%
Probable mannosyltransferase MNT2	protein glycosylation	Cell membrane	-	P46592	4	9.2%

3.4.3. Comparative proteomic analysis of *DSE1/dse1* strain grown under filamentous and non filamentous conditions

Comparative analysis has shown no unique peaks for the mutant strain grown under non filamentous conditions. 2% of the peaks were, however, unique to the mutant strain grown under filamentous conditions.

Candida database search was performed for data analysis and peaks identification. Unique peaks for each strain were compared to Candida theoretical mass spectrometric profiles. The best matches were considered to compute and infer peptide sequences with similar profiles, and to subsequently recognize a theoretical matching protein.

PMF analysis of the mutant strain grown under non filamentous conditions did not reveal any specifically expressed proteins that are absent in the mutant grown under filamentous conditions.

Table 6- List of proteins that are hypothetically exclusively expressed in the mutant strain grown under filamentous conditions (DSE f), not in the mutant strain grown under non filamentous conditions (DSE n)

DSE f – DSE n						
Protein name	**Protein Description**	**Cellular location**	**Accession number Protein name (CGD)**	**UniprotKB accession number**	**Number of peptide matches**	**% of coverage**
Probable mannosyltransferase MNT2	Protein glycosylation	Cell membrane	-	P46592	4	9.3%
pH-responsive protein 1	Hyphal growth	Cell membrane	CAL0002002. PHR1.	P43076	4	8.7%
Serine/threonine-protein kinase STE7 homolog	Virulence and morphological	-	-	P46599	3	6.5%
Serine/threonine-protein kinase BUR1	Pseudohyphal growth	nucleus	CAL0073553. CRK1.	Q9Y7W4	5	7.8%
Glucose-repressible alcohol dehydrogenase transcriptional effector	Filamentous growth	cytoplasm	CAL0002486. CCR4.	Q5A761	4	7.4%

Chapter Four

Discussion

C. *albicans* is a dimorphic fungus that can switch from the budding yeast cell form to the filamentous growth form (hypha). C. *albicans* virulence severity largely depends on this dimorphic transition: filaments support the penetration of tissue required for dissemination whereas yeast cells are essential for clonal development (Martchenko et al. 2004). Additionally, *C. albicans* possesses a broad arsenal of virulence factors contributing to its pathogenecity, maintaining cell wall integrity and overcoming host defenses (Naglik et al. 2003). *C. albicans* cell wall proteins were shown to be involved in virulence, antigenicity and filamentation, attributing a crucial function to *C. albicans* cell wall (Masuoka. 2004).

Since Dse1 is a cell wall protein, this study was conducted for the purpose of analyzing the cell wall proteome of *C. albicans DSE1/dse1* mutant. Chitin content and total cell wall protein content were assessed in the mutant and wild type strains, under both filamentous and non filamentous conditions. Cell wall proteins of both strains from both cell morphologies were analyzed using MALDI-TOF MS in order to generate unique mass spectrometric profiles, enlightening the existence of any possible mutation-specific, morphology-dependant protein expression.

The computed proteins that were considered significant in our study comprise cell wall proteins, as well as cytosolic proteins, since cytoplasmic contaminations are highly probable to occur, especially if the corresponding protein is highly upregulated and found in important amounts.

DSE1/dse1 heterozygous strain was previously characterized in our lab but the different phenotypes were observed only under non filamentous conditions. The strain

is however assumed to switch into its hyphal form once injected into the mice, thus increasing the virulence of the pathogen (Brown et al., 2007).

4.1. Comparison between the mutant and parental strains in their non filamentous form

The *DSE1/dse1* mutant strain grown under non filamentous conditions revealed a 16% decrease in total chitin content with respect to the WT strain. This can explain the reduced resistance of the mutant to the cell wall disrupting agent, calcofluor white, that targets chitin microfibril assembly (Daher et al.2011)

Hypothetically, PMF analysis suggests that the WT strain exclusively expresses the fungal-specific *CHS5* protein that is putatively involved in chitin biosynthesis (deduced by similarity, according to UniProtKB database). The lack of this protein expression in the mutant provides a satisfactory explanation of the observed decrease in chitin biosynthesis in the mutant. The decreased amount of chitin in the mutant cell wall may increase its permeability to SDS detergent that disrupts and solubilizes the plasma membrane, explaining the decreased resistance of the mutant to SDS.

Strain-specific protein expression suggested by PMF analysis can enlighten the discrepancies between the mutant and WT strains. The protein quantification assay showed a 14% decrease in total protein content of *DSE1/dse1* mutant strain with respect to the WT. The higher protein content in the WT compared to the mutant can be explained by the theoretical upregulation of proteins involved in protein biosynthesis like phenylalanyl-tRNA synthetase beta chain (Marcilla et al. 1998), ATP-dependent RNA helicase DED1 and Eukaryotic translation initiation factor 3 subunit C (both deduced by similarity, according to UniProtKB database).

The mutant also showed an increased sensitivity to hydrogen peroxide. That was explained by a probable altered architecture of the cell wall proteome due to the decreased amount of Dse1 protein, preventing the correct positioning of the proteins

responsible for countering oxidative stress damage. Interestingly, PMF analysis suggested a *DSE1/dse1*-specific expression of sorting nexin MVP1, a membrane protein having a role in cell communication, protein transport, and more importantly, phosphatidylinositol binding which is necessary for protein targeting and peripheral membrane localization (deduced by similarity, according to UniProtKB database). MVP1 may be involved in the alteration of cell wall protein positioning and organization, including those involved in combating oxidative stress, increasing the cell sensitivity to oxidative stress. Alternatively, PMF analysis revealed WT-specific expression of the Superoxide dismutase [Cu-Zn] SOD 1, a cytoplasmic antioxidant protein involved in the destruction of free radicals that are usually toxic to the cell (Hwang et al. 1999), thus protecting the WT against oxidative stress and promoting its pathogenecity.

Furthermore, the mutant strain exhibited a reduced ability to form biofilm and a delayed adhesion. PMF analysis showed the presence of Glucoamylase 1, a cell wall glycoprotein involved in carbohydrate metabolism and cell wall degradation-(polysaccharide degradation), changing the cell wall organization and decreasing biofilm formation (Sturtevant et al. 1999). Moreover, the WT-specific proteins included the pH-regulated antigen PRA1, a secreted glycoprotein that negatively regulates the host complement activation, evading and modulating by symbiont the host cell-mediated immune response, consequently increasing the pathogen adhesion to host cells (Soloviev et al. 2011). The absence of this protein in the mutant strain would make it more vulnerable to the host immune response and may delay its adhesion ability.

Also, the mutant was less virulent than the parental wild type strain. This was attributed to the defect in biofilm formation and the observed delay in adhesion (Daher et al. 2011). However, PMF analysis suggests a WT-specific expression of *ATG15,* a putative lipase (inferred by similarity, according to UniProt database), that was absent in the mutant-specific profile, thus might have rendered the WT strain more virulent as it allows the digestion of host membrane.

4.2. Comparison between the mutant and parental strains in their filamentous form

According to Daher et al., *DSE1/dse1* heterozygous strain exhibited a hyperfilamentous phenotype that was hypothesized to be due to an upregulation of a filamentation pathway. PMF analysis suggests the mutant-specific expression of the Serine/threonine-protein kinase CLA4, responsible for morphological switching and hyphal formation in *C.albicans* (inferred by similarity, according to UniProt database). Any probable upregulation of CLA4 might lead to the hyperfilamentous phenotype observed in the mutant strain, and absent in the WT.

The *DSE1/dse1* mutant strain grown under filamentous conditions revealed an 18% decrease in total protein content and 20% decrease in total chitin content with respect to the WT strain grown under filamentous conditions. Computed WT-specific proteins correlated with our protein and chitin quantification assays: PMF analysis showed the existence of Glucosamine-fructose-6-phosphate, mainly involved in amino-sugar and cell wall chitin biosynthesis (Smith et al. 1996). Similarly to the WT strain grown under non filamentous conditions, the WT filamentous form also expresses proteins that are involved in protein biosynthesis like ATP-dependent RNA helicase DED1 and Eukaryotic translation initiation factor 3 subunit C (both inferred by similarity, according to UniProt database).

The cell surface WT-specific 14-3-3 protein homolog can explain the increased virulence of the WT strain since it's involved in filamentous growth and pathogenesis (Palmer et al. 2004). This protein is down-regulated in the *DSE1/dse1* mutant, yielding to its reduced virulence.

4.3. Comparison between the filamentous and non filamentous forms of *DSE1/dse1* mutant

The mutant strain grown under filamentous conditions showed a 34% increase in total protein content compared to the mutant grown under non filamentous

conditions, whereas the difference in chitin expression was insignificant, limited to a slight increase of 3% in total chitin content in the mutant grown under filamentous conditions.

The protein quantification assay showed, however, about 20% increase in total protein content in the mutant grown under filamentous conditions with respect to the mutant grown under non filamentous conditions, possibly indicating that more proteins are expressed in the filamentous form. This can be further confirmed by PMF analysis where the *DSE1/dse1* heterozygous mutant grown under non filamentous conditions did not show any significant exclusiveness in protein expression, suggesting the presence of more proteins in the filamentous forms. The filamentous-specific proteins suggested through PMF analysis mainly comprise proteins involved in morphogenesis and hyphal growth, like the GPI-anchored cell membrane pH-responsive protein (Calderon et al. 2010), Serine/threonine-protein kinase STE7 homolog (inferred by homology, according to UniProt database) and Glucose-repressible alcohol dehydrogenase transcriptional effector (Uhl et al. 2003). As morphological switching and hyphal form are key factors for virulence, the exclusive expression of the cited proteins confers increased virulence and pathogenecity to the mutant grown under filamentous conditions.

Many of the proteins identified in this study explain the previously observed phenotypes. However, although PMF is an undeniable efficient tool for the identification of relatively pure proteins, it can fail to properly identify protein mixtures in complex samples, like the samples in our study. Also, many proteins are normally subjected to several modifications in the living cell, resulting in the additions of several groups to the original protein, thus varying its actual mass. The modifications dramatically modify the mass spectrometric profiles of proteins and they are hard to predict and locate. Hence, the modifications chosen in the search parameters are random and uncertain. We may have missed a specific modification or

a combination of several modifications that would have change the m/z values of some proteins.

Moreover, since proteins are enzymatically digested, a number of miscleavages may have occurred, yielding different peptides of different sizes and masses. These miscleavages cannot be predicted either, thus the mass spectrometric profiles of the experimental proteins can never be assumed to be entirely identical to the theoretical, fully-digested proteins in the databases.

In addition, the mass spectrometric profiles are compared to theoretical profiles in the corresponding databases that usually contain limited data. Therefore, a certain protein may exist in the sample, but the database can lack the corresponding mass spectrometric profile, leading to ambiguous conclusions.

Furthermore, the percentage of peptide matches leading to protein identification are always low, hence the identification can never be totally accurate. Besides, a long list of potentially matching proteins was obtained for each peak list, and we had to subjectively choose those that are relevant to our study. We may have missed an important protein, or considered one that is not really significant.

One additional useful assay could include running a "control database search" whereby all the peaks of each separate strain without substraction of any peaks are uploaded and compared against the *Candida* database. The resulting proteins can then be compared to the list shown here for a comprehensive overall observation of the proteins that are common to the different strains, or exclusive to one particular strain, and to verify the protein lists resulting from the unique strain- specific mass spectrometric profiles. Both resultant proteins should match. This was not feasible in this study since the complete peak list of each strain contained an average of 10500 peaks, whereas the searching tools have a maximum uploading capacity of 1000 peaks at once only.

Chapter Five

Conclusion and future perspectives

Mass spectrometry combined with peptide mass fingerprinting is a first identification step that helps recognizing unknown proteins and comparing proteomes. Many of the proteins identified in this study explain the previously observed phenotypes of *DSE1/dse1* mutant, like the reduced resistance to calcofluor white, increased permeability to SDS, increased sensitivity to hydrogen peroxide, hyperfilamentous phenotype and reduced virulence. Additionally, protein and chitin quantification assays results correlated with PMF analysis results to explain the differential protein expression and content in the different strains, as well as the mutant various phenotypes.

Nevertheless, various factors influence the outcome of the analysis and the suggested proteins require further experimental and statistical proofs.

More stringent identification techniques should be considered. A preparative effective step could have been the separation of complex protein samples by high-resolution two-dimensional gel electrophoresis or liquid chromatography (LC), followed by MALDI TOF (Thiede et al. 2005), in order to de-complex the samples before the analysis, thus minimizing the probability of identification errors. Additionally, more precise sequencing by MS/MS analysis can be used to confirm PMF-based protein identification (Damodaran et al. 2007).

Nevertheless, the separation of integral nonpolar membrane proteins by LC can also comprise ambiguity since LC separation may minimize the elution of long hydrophobic peptides (Da Silva et al. 2011), masking the presence of some membrane proteins that might be imperative in our study.

Moreover, considering that *C.albicans* cell wall proteins are subjected to several unpredictable post-translational modifications, the use of MS/MS might be limited

since undetermined PTM can dramatically change the matching outcomes, leading to possible misidentifications, whereas unsuspected PTM slightly affect the results of PMF analysis (Damodaran et al. 2007).

Alternatively, protein identification can be verified using western blotting, yet it is time-consuming and adequate antibodies are not always commercially available for all the investigated proteins, thus this technique might not be useful for studying a high number of samples (Damodaran et al. 2007).

In their article published in April 2011, Da Silva et al. suggested the combination and comparisons of PMF results obtained after MALDI-TOF MS and MALDI-FTICR MS analysis, after studying the effect of the different matrices on each instrument results. They concluded that the combined results obtained by TOF MS using DHB or CHCA and FTICR MS using DHB measurements reveal high levels of accuracy and that this methodology can be confident for an enhanced identification of various proteins in cell cultures.

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Chapter six

Appendix

6.1. List of additional WT- specific proteins that are absent in the mutant, both under non filamentous conditions, found through PMF

WT n – DSE n			
Description	**Accession number Protein name (CGD)**	**UniprotKB accession number**	**Number of peptide matches**
E3 ubiquitin-protein ligase BRE1	CAL0003779. BRE1.	Q5A4X0	5
ATP-dependent RNA helicase DBP4	CAL0004182. HCA4.	Q5AF95	4
Putative lipase ATG15	CAL0004893. ATG15.	**Q5A4N0**	4
Multiple RNA-binding domain-containing protein 1	CAL0000019. orf19.1646	**Q5AJS6**	5
Putative uncharacterized protein	CAL0004306. orf19.2797	**Q59PU6**	7
Protein HIR2	CAL0065931. orf19.11771	Q5AGM0	8
Chitin biosynthesis protein CHS5	CAL0002612. CHS5	O74161	4

6.2. List of additional mutant- specific proteins that are absent in the WT, both under non filamentous conditions, found through PMF

DSE n – WT n			
Description	**Accession number Protein name (CGD)**	**UniprotKB accession number**	**Number of peptide matches**
Phospho-2-dehydro-3-deoxyheptonate aldolase, phenylalanine-inhibited	-	P34725	5
ATP-dependent RNA helicase DBP2	CAL0003204. DBP2	Q59LU0	6
SWR1-complex protein 4	CAL0005735. orf19.7492	Q5AAJ7	5
Neutral trehalase	-	P52494	4

Heat shock protein 90 homolog	CAL0003079. HSP90	P46598	9
Mitochondrial homologous recombination protein 1	CAL0005665. orf19.439	Q5A2A2	5
Chromatin modification-related protein EAF1	CAL0001486. VID21	Q5A119	8
Crossover junction endonuclease MUS81	CAL0000062. orf19.4206	Q59NG5	7
potential spliceosomal U2AF large subunit	-	68481460 (NCBI database)	10
potential nuclear cohesin complex SMC ATPase fragment	-	68471834 (NCBI database)	8

6.3. List of additional WT- specific proteins that are absent in the mutant, both under filamentous conditions, found through PMF

WT f - DSE f			
Description	**Accession number Protein name (CGD)**	**UniprotKB accession number**	**Number of peptide matches**
tRNA (guanine-N(7) methyltransferase	CAL0001314. orf19.3798	Q5A692	4
ATP-dependent RNA helicase DBP4	CAL0004182. HCA4.	Q5AF95	6
SEC14 cytosolic factor (Phosphatidylinositol/phosphatidylcholine transfer protein)	CAL0003685. SEC14.	P46250	3
Phosphatidylinositol 3-kinase VPS34	-	Q92213	6
Eukaryotic translation initiation factor 3 subunit B	CAL0003307. PRT1.	Q5AGV4	3
DNA repair protein RAD5 (DNA damage/repair)	CAL0004569. orf19.2097.	Q5ACX1	6
Conserved oligomeric Golgi complex subunit 6 (Protein transport)	-	Q59MF9	6
Protein PDC2 (Essential for the synthesis of pyruvate decarboxylase)	-	O60035	4
Heat shock protein 78, mitochondrial (Stress response)	CAL0063839. HSP78.	Q96UX5	8

Potential ARF GAP (regulation of ARF GTPase activity)	CAL0001428. AGE1.	Q5AI02	8

6.4. List of additional mutant- specific proteins that are absent in the WT, both under filamentous conditions, found through PMF

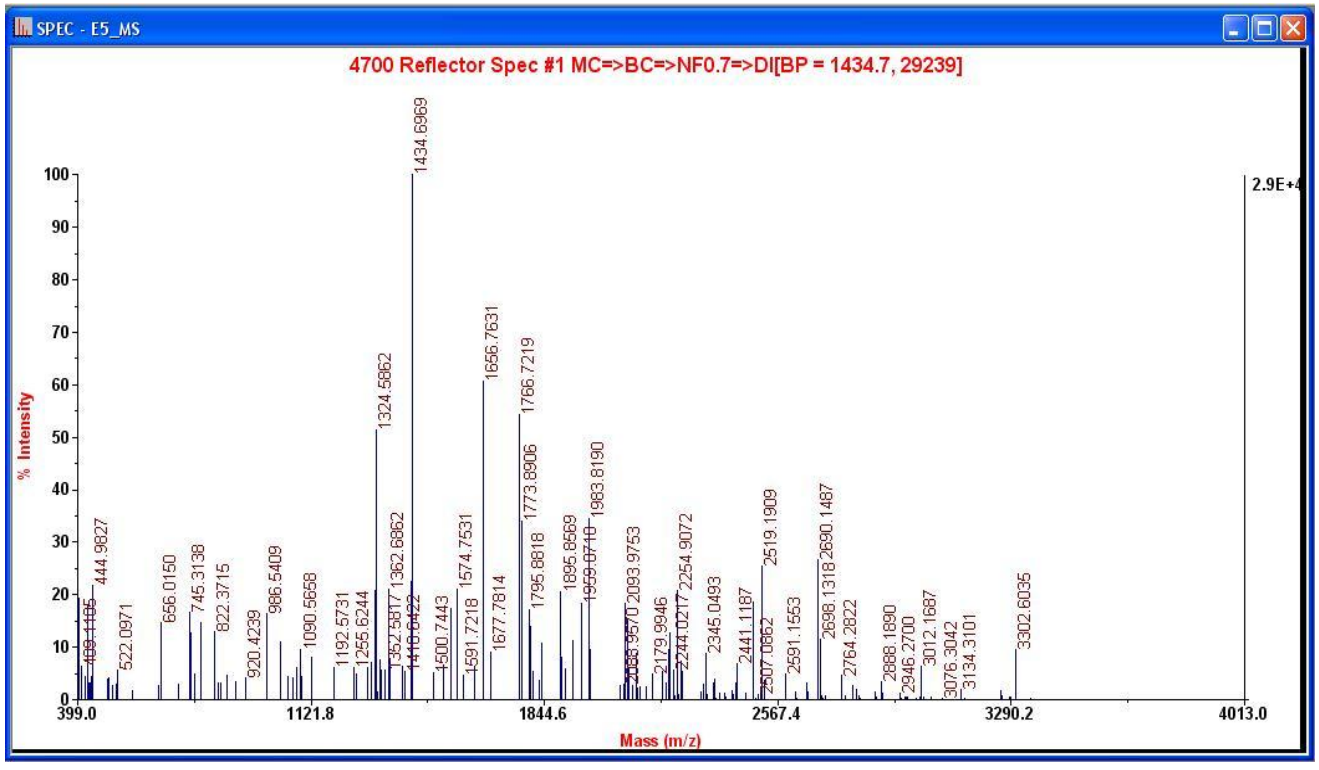
DSE f – WT f			
Description	**Accession number Protein name (CGD)**	**UniprotKB accession number**	**Number of peptide matches**
Tethering factor for nuclear proteasome STS1 (Involved in ubiquitin-mediated protein degradation.)	CAL0005386. orf19.4849.	Q5APB6	4
Altered inheritance of mitochondria protein 23, mitochondrial	CAL0000393. orf19.6982.	Q59YU1	3
Transcription initiation factor TFIID subunit 4 (response to drug)	-	Q59U67	7
Chromatin modification-related protein EAF7 (DNA repair)	CAL0005877. orf19.497.	Q5A6Q7	3

6.5. List of additional mutant-specific proteins, expressed under filamentous conditions and absent in the non filamentous growth

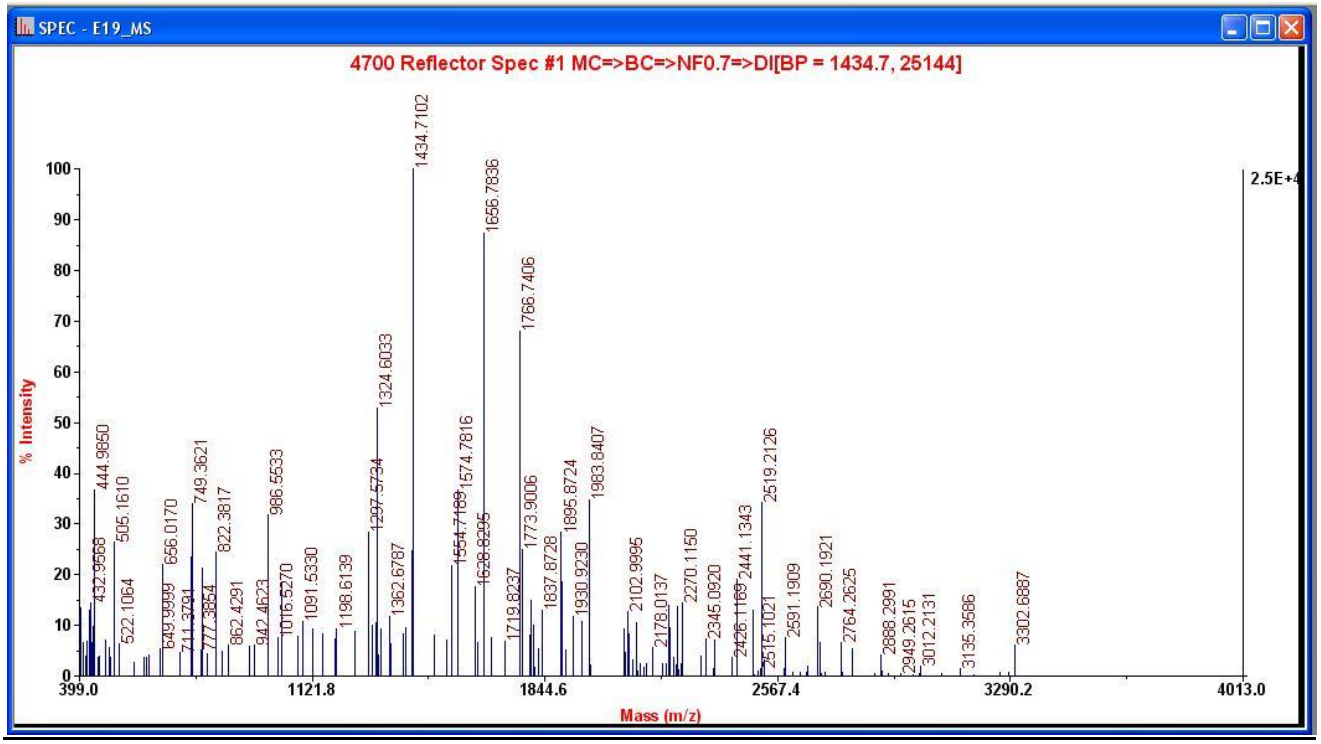
DSE f – DSE n			
Description	**Accession number Protein name (CGD)**	**UniprotKB accession number**	**Number of peptide matches**
Potential very long-chain fatty acyl-CoA synthetase Fat1p	CAL0000418. FAT1.	Q59NN4	5
Potential polyamine N-acetyl tranferase (GNAT family). Has domain(s) with predicted N-acetyltransferase activity and role in metabolic process	CAL0004655.orf19.1465.	Q5ALW1	3
Potential alpha-1,6-mannanase. Catalytic activity.	CAL0000901. orf19.1765.	Q59XY6	3
Putative lipase ATG15 (membrane, among other locations)	CAL0004893. ATG15.	Q5A4N0	4
Acetyl-coenzyme A synthetase 1 (AMP binding. ATP binding. acetate-CoA ligase activity)	-	O94049	4

ATP-dependent RNA helicase DBP4	CAL0004182. HCA4.	Q5AF95	6
Protein HIR1 (chromatin modification)	CAL0004571.orf19.2099.	Q5ACW8	4
Phosphatidylinositol 3-kinase VPS34 (phosphatidylinositol-mediated signaling)	-	Q92213	5
UDP-N-acetylglucosamine pyrophosphorylase	-	O74933	3
Mitochondrial Rho GTPase 1 (can be found in cell membrane. Mitochondrial Rho GTPase 1(	-	Q5ABR2	4
GTPase GUF1 (transmembrane. GTP binding)	CAL0005338. orf19.5483.	Q59P53	6
Vacuolar-sorting protein SNF7 (hyphal growth - response to drug and pH-pathogenesis)	CAL0005125. SNF7.	Q5ABD0	5

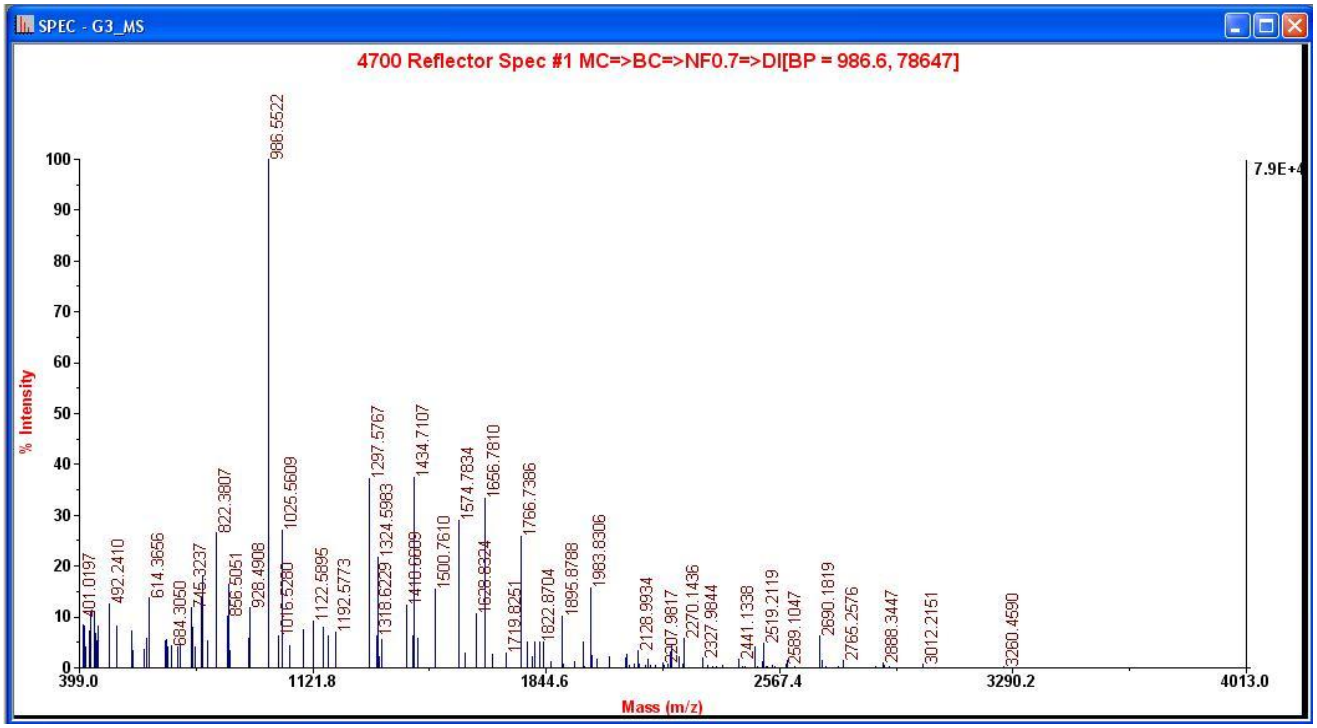
6.6. Mass spectrum corresponding to the WT strain under non filamentous conditions



6.7. Mass spectrum corresponding to the WT strain under filamentous conditions



6.8. Mass spectrum corresponding to the mutant strain under non filamentous conditions



6.9. Mass spectrum corresponding to the mutant strain under filamentous conditions