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FACULTY OF PHARMACY
DEPARTMENT OF MICROBIOLOGY II

DIPLOMOVÁ PRÁCA
DIPLOMA THESIS

Detection of Sap2 in the secretome of *Candida albicans* cell wall and secretory mutants

Detekcia Sap2 proteínu v sekretóme kmeňov *Candida albicans* mutantných v bunkovej stene a sekrécii

Supervisors: Prof. Gloria Molero, Prof. Lucía Monteoliva
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Madrid 2017 **Kollárová Nikola**

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"I would like to thank all girls in the laboratory for teaching me everything, always advising me and answering every question I had, especially Ahinara Amador García. She was also very willing to help me with my thesis. A big thank belongs to Dr. Gloria Molero, Lucía Monteoliva and Concha Gil for all meetings, advices and ideas how to continue and improve all the methods. Big thanks to my supervisor PharmDr. Ondřej Janďourek, PhD., for helping me with the formal aspects of my diploma thesis."

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2 ABSTRACT

Candidate: Nikola Kollárová

Title of diploma thesis: Detection of Sap2 in the secretome of *Candida albicans* cell wall and secretory mutants

Charles University, Faculty of Pharmacy in Hradec Králové, Department of Biological and Medicinal Sciences

Complutense University of Madrid, Faculty of Pharmacy, Department of Microbiology II

Study program: Pharmacy

Backgound: The aim of this diploma thesis was to search for *C. albicans* proteins involved in the secretion of the secreted aspartyl proteinase 2 enzyme (Sap2) evaluating the ability to degrade BSA (bovine serum albumin) as a source of nitrogen in several cell wall and secretory mutants of *C. albicans.* The work was carried out at the Department of Microbiology II, Faculty of Pharmacy, Complutense University of Madrid.

Methods: The supernatant samples of several *Candida albicans* mutants were tested by SDS-PAGE electrophoresis and stained. Bands corresponding to BSA were observed and compared to controls. The other method was counted with 96-well plate.

Results: The correlation between optical density and degradation of BSA was observed. Some mutants with disability to degrade BSA were found in a pilot screening of the ability to degrade BSA using 96-well plate method. That fact was confirmed by SDS-PAGE electrophoresis. *C. albicans* mutants showing this defect, that was proved by both methods, were *ecm33*Δ, *kex2*Δ, *ypt72*Δ, *orf*.19.1567Δ and *pbs*2Δ.

Conclusions: The mutants with disability to degrade BSA as a sole source of nutrients gradually died in liquid YCB-BSA medium and their OD were considerably lower than in the other cases. It was possible to confirm this absence of degradation by SDS-PAGE electrophoresis. Assumption was that mutants with disability to degrade BSA had problems with Sap2 secretion. This fact was confirmed by western blot using Sap2 antibody.

KEYWORDS: *C. albicans*, mutant, optical density, bands, electrophoresis, supernatant, BSA, degradation, Sap2, cytoplasmic extract.

3 ABSTRAKT

Kandidát: Nikola Kollárová

Názov diplomovej práce: Detekcia Sap2 proteínu v sekretóme kmeňov *Candida albicans* mutantných v bunkovej stene a sekrécii

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Študijný program: Farmácia

Cieľ práce: Cieľom tejto diplomovej práce bolo hľadať proteíny *C. albicans* podieľajúce sa na sekrécii Sap2 enzýmu vyhodnotením schopnosti degradovať BSA ako zdroj dusíka u niektorých kmeňov *C. albicans* mutantných v bunkovej stene a sekrécii. Práca bola vykonaná na Department of Microbiology II, Faculty of Pharmacy, Complutense University of Madrid.

Metódy: Vzorky supernatantov niekoľkých mutantov *C. albicans* boli testované SDS-PAGE elektroforézou a zafarbené. Na geli boli pozorované prúžky odpovedajúce BSA a porovnané s kontrolami. Druhá metóda bola vyhodnotená s 96-jamkovými doskami.

Výsledky: Bola zistená korelácia medzi absorbanciou a degradáciou BSA. V pilotnom skríningu, kde bola testovaná schopnosť degradovať BSA pomocou metódy s 96-jamkovými doskami boli nájdení niektorí mutanti, ktorí nemali schopnosť degradovať BSA. Tento fakt bol potvrdený SDS-PAGE elektroforézou. Mutanti *C. albicans* s týmto defektom, čo bolo dokázané obomi metódami, boli *ecm33*Δ, *kex2*Δ, *ypt72*Δ, *Orf*.19.1567Δ and *pbs*2Δ.

Závery: Mutanti, ktorí neboli schopní degradovať BSA, ako jediný zdroj výživy v tekutom YCB-BSA médiu postupne umierali a ich absorbancia bola tak značne nižšia ako v ostatných prípadoch. Výsledok bolo možné potvrdiť pomocou SDS-PAGE elektroforézy. Predpoklad bol, že mutanti, ktorí nie sú schopný degradovať BSA, majú problémy so sekréciou Sap2. Tento fakt bol potvrdený metódou western blot s použitím Sap2 protilátky.

Kľúčové slová: *C. albicans,* mutant, optická hustota, prúžok, elektroforéza, supernatant, BSA, degradácia, Sap2, extrakt cytoplasmy.

4 LIST OF ABBREVIATIONS

AIDS	acquired immune deficiency syndrome
ALS	agglutinin-like sequence
BSA	bovine serum albumin
CGD	*Candida* genome database
cyt	cytoplasmic extract
dH_2O	deionised water
DNA	deoxyribonucleic acid
DTT	dithiothreitol
ER	endoplasmic reticulum
ESCRT	endosomal sorting complex required for transport
GPI	glycosyl phosphatidyl inositol
HIV	human immunodeficiency virus
HOG	high-osmolarity glycerol
kDa	kilodalton
Lip	lipase
MAP	mitogen-activated protein
MVB	multivesicular body
OD	optical density
O/N	overnight
PAGE	polyacrylamide gel electrophoresis
PBS	phosphate-buffered saline
Plb	phospholipase
PM	protein marker
PMSF	phenylmethanesulfonylfluoride

PSA	ammonium persulphate
rpm	revolutions per minute
Sap	secreted aspartyl proteinase
SD	synthetic, fully defined
SDS	sodium dodecyl sulphate
SNARE	soluble *N*-ethylmaleimide-sensitive factor attachment protein receptor
SPC	signal peptidase complex
sup	supernatant
TEM	transmission electron microscope
TEMED	tetramethylethylenediamine
UDP	uridine diphosphate
WB	western blot
YCB	yeast carbon base
YNB	yeast nitrogen base
YPD	yeast extract peptone dextrose

5 THE ASSIGNMENT OF DIPLOMA THESIS - THE AIM OF THE WORK

I will follow up the scientific work *Global Proteomic Profiling of the Secretome of Candida albicans ecm33 Cell Wall Mutant Reveals the Involvement of Ecm33 in Sap2 Secretion* where Ana Gil-Bona and her team observed defect in secretion of Sap2 in the *C. albicans* cell wall mutant *ecm33Δ,* called RML2U, but not a general defect in the secretory pathway, as the secretion of other proteins was not impaired (Gil-Bona *et al.*, 2015b).

The aim of my work was to find possible *C. albicans* mutants that were unable to secrete Sap2 enzyme, which is responsible for degradation of BSA as a sole source of nitrogen. I was detecting Sap2 in the secretome of *C. albicans* mutants in order to see if that disability is specific only to *ecm33Δ* mutant or it is connected to defects in cell wall structure.

For that purpose, I have used a selection of mutants with defects in proteins related to glucan metabolism, mannoproteins, chitin and also mutants with defects in the protein secretory pathway that were defective in at least one of these processes: transport, organisation of organelles, protein folding and vesicle-mediated transport.

6 INTRODUCTION

Candida albicans is a yeast, which grows normally on skin and mucosae of a big number of healthy people. It exists in balance with many host factors such as pH, hormones and other microorganisms of the microbiota. When this balance is deflected *C. albicans* may cause infections. These infections range from mucocutaneous to disseminated forms, and are especially dangerous for immunocompromised people such as HIV/AIDS (Human Immunodeficiency Virus/Acquired Immune Deficiency Syndrome) positive patients, people with transplanted organs or those who take corticosteroids or other immunosuppressants.

To study the functions of the different *Candida* proteins, several mutants have been obtained and the consequences of the defect have been studied. The defect can cause disruptions of many processes, sometimes including virulence. One of those processes is the secretion of the major secreted aspartyl proteinase Sap2 that is a virulence factor and is also responsible for BSA (bovine serum albumin) degradation, which is usually used as source of nitrogen. If *C. albicans* has problems with Sap2 secretion, it cannot survive in YCB-BSA (yeast-carbon base, bovine serum albumin) culture medium.

7 THEORETICAL PART

7.1 *Candida albicans* and candidiasis

C. albicans is an asexual, diploid, aerobic and polymorphic (ability to grow in several morphologies) fungus. It is a common constituent of the microbiota in gastrointestinal tract, skin, oral cavity and reproductive system under normal conditions (Nobile *et al.*, 2015). When a child is born and parturition is conducted through the birth canal his or her skin and the digestive system is naturally colonised. This fact is considered as a disadvantage in children born by C-section (Jones, 1990).

There is a balance between *C. albicans* and other factors in body with healthy immune system. However, if there is a disruption of this balance, *C. albicans* can overgrow and lead to an infection called candidiasis. Important factors for its occurrence are pH of local area, stress, state of immune system, antibiotic or immunosuppressant therapy, infection caused by another microorganism, food composition and many others.

The infection can be local or systemic. Local candidiasis such as vulvovaginal, oropharyngeal candidiasis or thrush can be usually easily treated with topical drugs. Approximately 75% of women experience vulvovaginal candidiasis once per their life and 5–10% of them suffer from chronic episodes (Sudbery, 2011). However, one of the biggest problems is the occurrence of nosocomial (hospital) infections. *C. albicans* is the most common etiologic agent of invasive candidiasis. It is the fourth leading cause of septicaemia with 50% mortality (Wisplinghoff *et al.*, 2004). This can happen for example in patients placed in intensive care units when *C. albicans* gets to the bloodstream and causes candidemia. In addition, it can attack more internal organs and can lead to a serious problem. Systemic infections (such as endocarditis, sepsis or meningitis) are serious life-threatening illnesses. It is dangerous especially for immunocompromised patients with chemotherapy, AIDS or patients with immunosuppressive therapy like corticosteroids (Nobile *et al.*, 2015). Among other predisposing factors belong burns, neutropenia, endocrinopathy, parenteral administration of drugs, catheterization, age (newborns or on the contrary seniors) or illnesses like diabetes mellitus (Buchta *et al.*, 2002).

The genus *Candida* involves over 100 species, from which *Candida albicans* is the most predominant cause of candidiasis infections (63–70%), followed by *C. glabrata*

(44%), *C. tropicalis* (6%) and *C. parapsilosis* (5%). *C. tropicalis* is the most virulent pathogen connected with colonisation and invasive diseases. It affects gastrointestinal system in patients with neutropenia and broad spectrum antibiotic users (Dyavaiah *et al.*, 2013, Bednář et. al., 1999).

There are other fungal infections such as aspergillosis (the second most common nosocomial fungal infection), cryptococcosis, pneumocystosis dermatomycoses, zygomycoses and the others less abundant diseases (Beneš *et al.*, 1999).

7.2 Diagnosis and treatment of candidiasis

C. albicans causes wide range of infections from superficial mucocutaneous to invasive candidiasis with dissemination. White patches and itching are typical for superficial candidiasis, for vulvovaginal candidiasis it is irritation, white vaginal discharge and an oedema. Pneumonia caused by *C. albicans* can be caused by aspiration or by haematogenous dissemination. The diagnosis of pneumonia requires a histopathology section from pulmonary tissue. An invasive candidiasis includes candidemia, endocarditis, joint and bone infections, and endophthalmitis. Disseminated diseases may manifest themselves by skin lesions which can be painless, erythematous or pustular to macronodular (Pappas *et al.*, 2003).

An appropriate diagnosis allows adequate therapy. The mucocutaneous infections are usually easily diagnosed; however, there is no specificity in the clinical manifestations of disseminated fungal infections. The problem is that the blood culture isolation of fungi may be negative. The direct evidence (microscopy, histology or cultivation of biological material in a growth media) of the disease-causing agent is required (Beneš *et al.*, 2009, Sobel *et al.*, 2000).

Fungi are diagnosed by microscopy making use of native, caustic mounts or different staining methods. Mostly used staining methods are method according to Gomori (revealing glucan and chitin), periodic acid Schiff technique (showing polysaccharides in the cell wall), staining with calcofluor dye for fluorescent microscopy or Gram staining (Beneš *et al.*, 2009).

There are other types of screening for diagnosis of *C. albicans:* the serologic methods that use antigens and antibodies. The disadvantage is the lack of antibody response in immunocompromised patients and cross-reactions resulting in false positivity

because antibodies are also present in colonized healthy individuals (Sulahian *et al.*, 1996, Sobel *et al.*, 2000).

It is important to distinguish systemic infection from local one. It is called an invasive candidiasis when *Candida* spp. penetrates the tissues below epithelial surface or infect the viscera (Sobel *et al.*, 2000). Treatment is always based on antifungal agents. The therapy depends on the type of infection. Local infection can be quite easily treated with topical forms of drugs. On the other hand, systemic candidiasis are treated by intravenously administered drugs because the patients who suffer from systemic candidiasis should be hospitalised (Beneš *et al.*, 2009).

There were only few antifungal agents like amphotericin B and azole agents, in the past. Nowadays, there are substances with different spectrum, side effects and mechanisms of action (Owens *et al.*, 2010). Many of these mechanisms are based on the fact that the main sterol of cytoplasmic membrane is ergosterol. It is the essential difference between fungal and mammalian cells (Beneš *et al.*, 2009).

One of the classification of antifungal drugs is based on chemical structure.

- Polyenes (Amphotericin B, nystatin, natamycin)
- Azoles (imidazoles, triazoles)
- Anti-metabolites (flucytosine)
- Echinocandins (caspofungin)
- Allylamines (terbinafine)
- Morpholines (amorolfine)
- Griseofulvin

Mechanisms of action can be also one of the classification aspects (Figure 1).

- Damage of the cell membrane (polyenes, azoles, allylamines and morpholine).
- Inhibition of the cell wall synthesis (echinocandins and pneumocandins).
- Inhibition of proteosynthesis and partially DNA synthesis (deoxyribonucleic acid) in fungal cell (anti-metabolites).
- Inhibition of mitosis (griseofulvin).

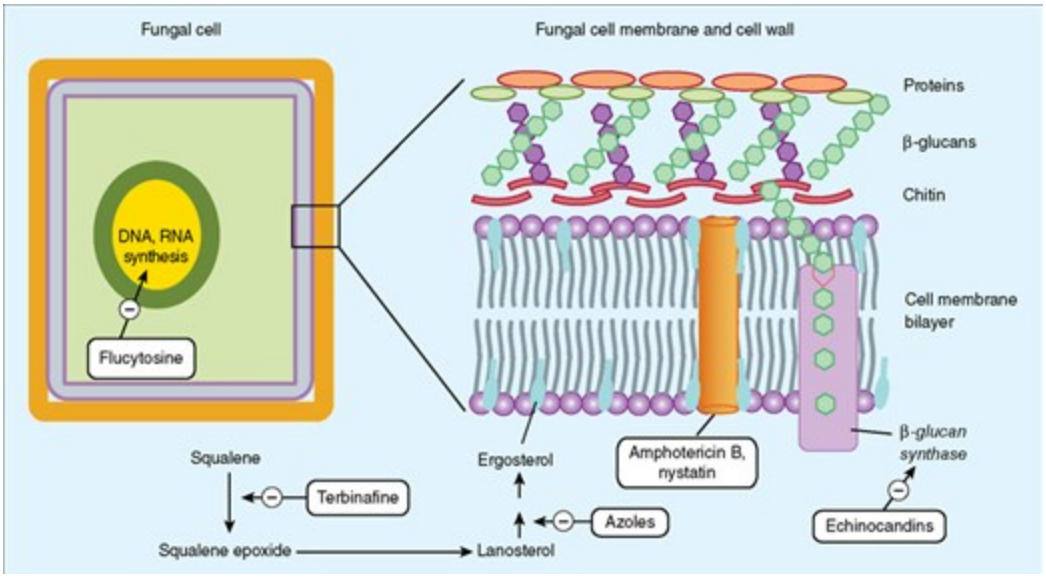


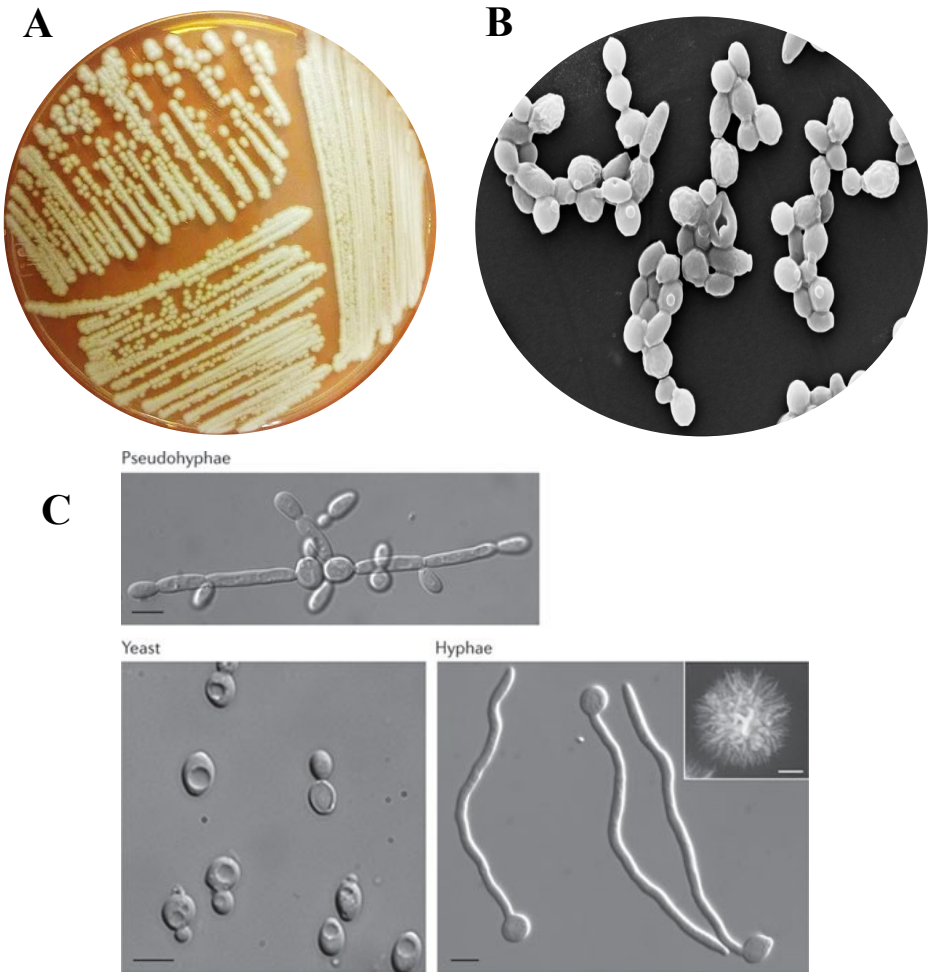
Figure 1: Mechanisms of action of antifungal drugs.

Adapted from (Web 1 – https://sheerazgul.wordpress.com/2013/12/27/expected-ospe-slides-for-pharmacology).

The first choice agent is an azole antifungal drug called fluconazole (for systemic, skin, mucous, and vaginal mycoses). It is an agent which can be administered intravenously and orally too. In local infections are used topical antifungals (nystatin and clotrimazole) whilst for systemic ones other systemic antifungal drugs (amphotericin B, voriconazole, itraconazole, caspofungin and flucytosine) can be administered (Buchta *et al.*, 2002). Amphotericin B has shown severe side effects (nephrotoxicity) whereas fluconazole is well tolerated by patients. The problem is connected with lot of azole resistant strains (Beneš *et al.*, 2009). It can be solved using lipidic carriers, which are used in order to reduce the toxicity of amphotericin B (liposomes, cholesterol carriers or colloid dispersions) (Rozsypal, 2008).

7.3 Morphology

This microorganism is highly variable and adaptable. It is able to transform into many morphological phenotypes such as budding yeast-form cells (blastospores), filamentous forms (pseudohyphae and true hyphae) and chlamydospores (Figure 2). The importance of yeast form is in colonisation and hyphae form during adhesion and invasion (Staniszewska *et al.*, 2012, Monteoliva *et al.*, 2011). However the function of pseudohyphae is still unclear. Chlamydospores are formed only in certain conditions (Mayer *et al.*, 2013).



Scale bars-5 µm, the inset on the hyphae panel-1mm.

Figure 2: Macroscopy of *C. albicans* colonies in YPD media – enlargement (A), microscopy of *C. albicans* yeast cells – scanning electron microscopy (B) and morphological forms of *C. albicans* – phase contrast microscopy (C).

Author Nikola Kollárová, Universidad Cumplutense Madrid, Faculty of Pharmacy, Department of Microbiology II (A), adapted from (Web 2 – http://www.biotrans.uni.wroc.pl/en/galeria,4,badania.html) (B) and (Sudbery, 2011) (C).

Normally *C. albicans* yeast cells grow from four to six μm and their reproduction is represented by budding. It is able to grow in common culture media. It takes one to three days on solid media to grow as smooth, white or cream, glistening colonies. The optimal range of pH for its existence is very wide ranging from 2.5 to 7.5.

Hyphal form is created by prolongation of daughter cell without division from mother cell. The transformation between yeast and hypha can be induced by changes in temperature, pH, amount of CO_2, starvation and various compounds such as *N*-acetylglucosamine or proline in culture media at low cell densities (‹10^7 cells/mL). Reverse transfer from hyphal to yeast form is promoted by lower temperature (‹37°C), acidic pH, higher concentration of glucose, and high cell densities (›10^7 cells/mL) (Maria Teresa Mascellino, 2013). Although the inability to form hyphae is generally connected to weakened virulence in mutants of *C. albicans*, both forms are important for pathogenicity. Hyphal forms are more important for the tissue invasion and the yeast form helps the dissemination (Mayer *et al.*, 2013, Buchta *et al.*, 2002).

7.4 Cell wall

Cell wall provides protection from environmental conditions such as temperature, osmotic and oxidative stress. It gives the shape and strength to yeasts. It is the first line defence in interaction with host, thus it is important for virulence and pathogenicity (Gil-Bona *et al.*, 2015a). It carries important antigen factors and provides yeast the ability of adhesion. The components of cell wall are bound by different kind of bonds (covalent, hydrogen, salt-type associations, hydrophilic or hydrophobic interactions) (Free, 2013).

The layered structure of cell wall consists of carbohydrates (mannan, β-glucans and chitin) which represent 80–90% of its composition, cell-wall proteins (6–25%) and lipids (1–7%). β-glucans and mannan create frame of the cell (Figure 3). Hyphae contain three times more chitin than yeasts themselves. Glucan and chitin appear to be more concentrated in the inner layer of the cell-wall. On the other hand, proteins and mannoproteins are mostly situated in the outer layer (Gil-Bona *et al.,* 2015a, Klis, 1994). When the integrity of cell wall is disrupted it may results in sensitivity to environmental conditions and in changes of growth, morphology and viability (Munro *et al.*, 2001).

Hydrophobic character of the yeast cells enables better adherence to tissues and in addition higher resistance to phagocytes. *C. albicans* is the only fungus which is able to change its hydrophobicity depending on conditions and the stage of growth. This ability is obviously connected with conformational changes of the mannoprotein fibrils (Masuoka *et al.*, 2004).

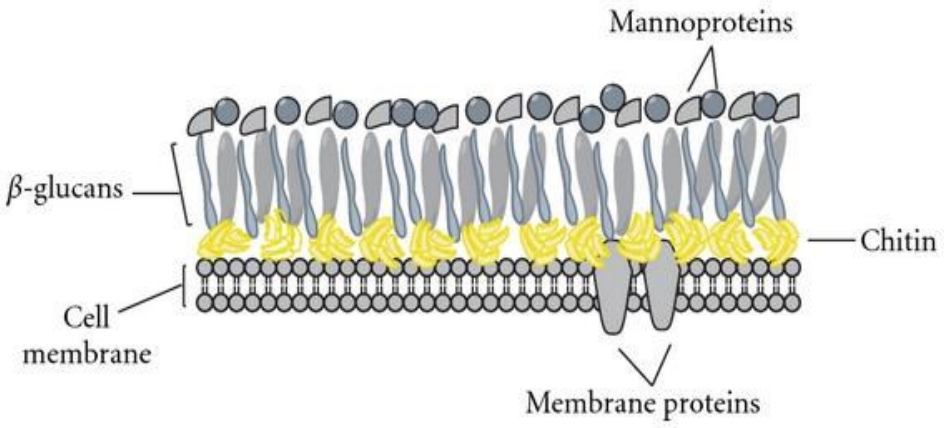


Figure 3: Cell-wall composition of *C. albicans*.
Adapted from (Web 3 – http://www.holistichelp.net/blog/is-lufenuron-safe-and-effective-for-candida/).

7.4.1 Glucans

There are only β-glucans in yeast cell wall. β-glucan represents approximately 40% of cell wall composition in yeasts thus it is the most abundant polysaccharide and its amount increases twice in hyphae (Free, 2013). Glucans are polymers of glucose. There are two types of glucan: β-(1,3)-glucan and β-(1,6)-glucan (Figure 4). Glucans and complexes of β-glucan and mannans are obviously released into bloodstream of infected patients. They are able to cause anaphylactic shock in murine models because of the toxicity of these compounds. *C. albicans* glucans suppress the activity of monocytes directly and T-cells indirectly. That is the reason why scientists suggest that they play role in the development of candidiasis.

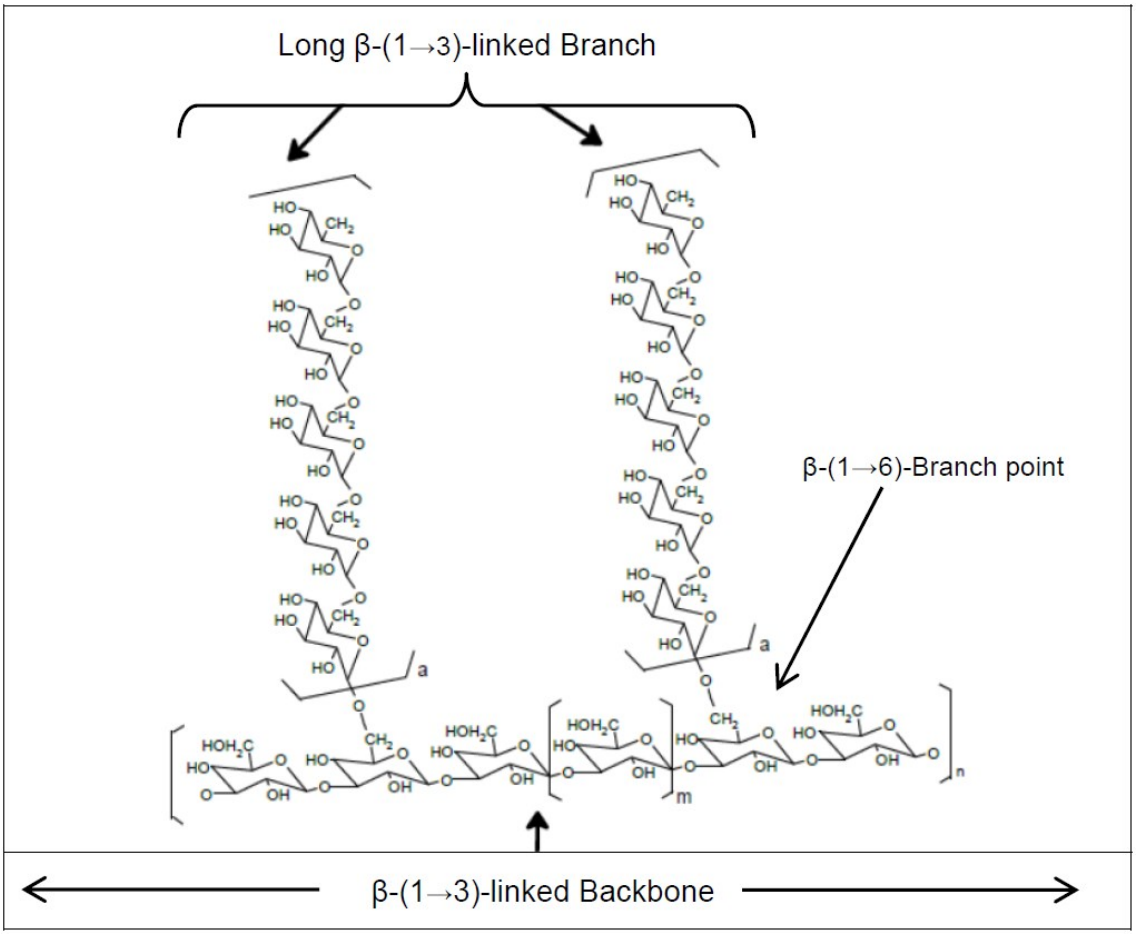


Figure 4: Chemical structure of two types of glucan present in *C. albicans* cell wall. Adapted from (Web 4 – https://www.intechopen.com/books/lipid-metabolism/spent-brewer-s-yeast-and-beta-glucans-isolated-from-them-as-diet-components-modifying-blood-lipid-me).

There are glucan-specific receptors on cells of the immune system. The synthesis of glucans involves transglycosylation reactions of glucosyl residues from UDP-glucose (uridine diphosphate). The major groups of cell wall proteins are attached to β-(1,6)-glucan by glycosylphosphatidylinositol (GPI) residue or to chitin through β-(1,3)-glucan linker (Munro *et al.*, 2001).

7.4.2 Chitin

Chitin is a linear polysaccharide composed of over than 2000 units of *N*-acetylglucosamine (GlcNAc) (Figure 5). It is covalently connected to β-(1,3)-glucan. Synthesis of chitin is performed by chitin synthases. These enzymes are situated in cytosol in special vesicles called chitosomes, which ensure transfer of these enzymes to the place of synthesis. Chitin synthase type two is the most abundant enzyme *in vitro*.

However, type 3 provides the synthesis of majority of chitin *in vivo*. Type one is unique and important for virulence, cell integrity and it is involved in septum formation (Ruiz-Herrera *et al.*, 2005, Gilbert *et al.*, 2010).

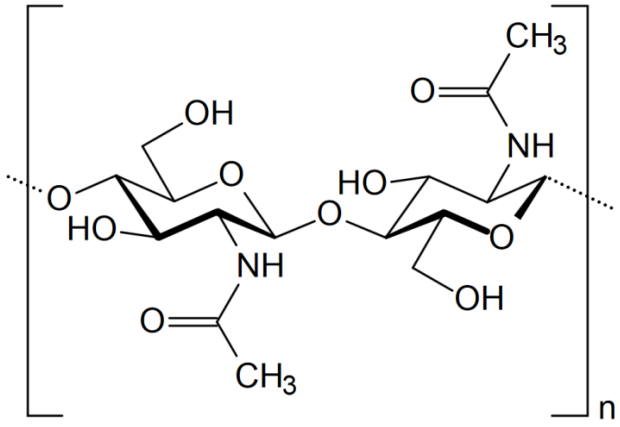


Figure 5: Chemical structure of chitin.
Adapted and edited from (Younes *et al.*, 2015).

7.4.3 Mannans

The outer layer is made by mannans, which create 40% of polysaccharides, covalently bounded with proteins. Lipids constitute a very little part of candida's cell wall and of fungi in general (Ruiz-Herrera, 2005). Phospholipomannan is one of the most interesting lipid. It seems to play a crucial role in protection, signalling and adhesion (Free, 2013).

7.5 Pathogenicity

C. albicans is able to attack different hosts because of a wide range of virulence factors. They include transition between yeast and hyphal forms, expression of adhesins, invasion into epithelia and endothelia, biofilm formation, phenotypic switching and secretion of hydrolytic enzymes. Very important is also the ability of quick adaptation to different environmental conditions due to its metabolic flexibility and the stress response system (Figure 6).

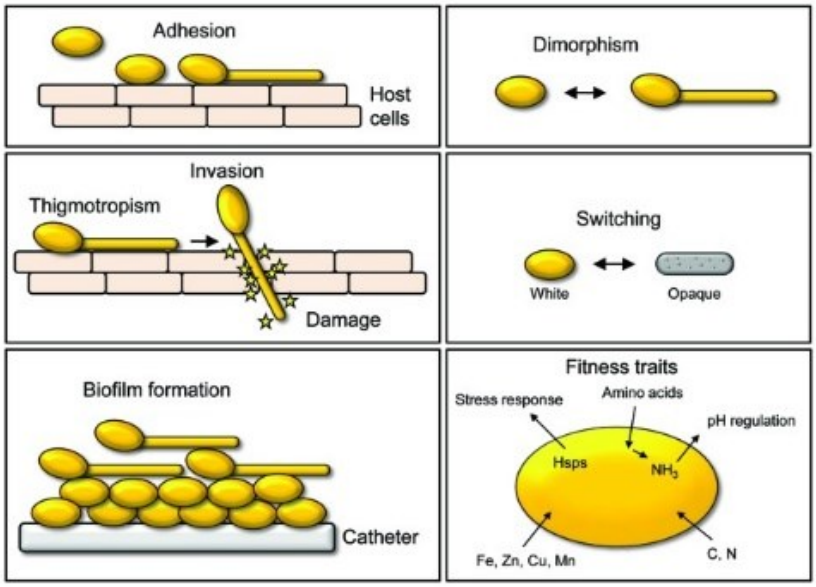


Figure 6: Pathogenicity mechanism of *C. albicans*.
Adapted from (Mayer *et al.*, 2013).

7.5.1 Adhesion and invasion

There is a special group of proteins called adhesins. They provide the ability to adhere to other *C. albicans* cells, other microorganisms, host cells and abiotic surfaces. The best known adhesins are agglutinin-like sequence (ALS) proteins. Eight members belong to this family of adhesins (Als1–7 and 9). Genes for these proteins encode GPI-linked cell surface glycoproteins. Als3 is typical for adhesion and its gene is upregulated during *in vitro* oral infection and during *in vivo* vaginal infection. Other important adhesin is Hwp1 (hypha-associated GPI-linked protein) which ensures covalent link of hypha to host cell. Mutants in this gene (*hwp1Δ/Δ*) have lower adhesion capacity and lower virulence in mouse models. There are also morphology-independent proteins that play a role in adhesion too: the GPI-linked proteins Eap1, Iff4 and Ecm33; the non-

covalent wall-associated proteins Mp65, a putative β-glucanase and Phr1, a β-1,3-glucanosyl transferase; the cell-surface associated proteases: Sap9 and Sap10 and the integrin-like surface protein Int1.

C. albicans uses two mechanisms to invade host cells, namely active penetration and induced endocytosis. Invasins, such as E-cadherin on epithelial cells and N-cadherin on endothelial cells, are cell-surface proteins important for induced endocytosis, which are mediating binding to host ligands. There are two invasins: Als1 (which is an adhesin too) and Ssa1 (a member of heat shock protein 70 family). On the other hand, active penetration requires viable *C. albicans* hyphae. Saps are probably also involved in active penetration but lipases and phospholipases are not (Mayer *et al.*, 2013).

7.5.2 Biofilm formation

The most common substrates for *Candida* to form biofilms are catheters, dentures (abiotic) and mucosal cell surfaces (biotic). This process includes adherence of yeast cells to substrates, proliferation of these yeasts, formation of hyphae in the upper part of biofilm, accumulation of extracellular matrix and dispersion of yeasts from the biofilm complex. Resistance to antimicrobial agents is higher in mature biofilms. It is because of its architecture, biofilm matrix, raised expression of efflux pumps and metabolic plasticity. Hsp90 (heat shock protein 90) is a key regulator of dispersion in *C. albicans* biofilms and it is responsible for antifungal drug resistance (Mayer *et al.*, 2013).

Production of biofilm matrix, where the major part is β-(1,3)-glucan, is regulated negatively by the zinc-responsive transcription factor (Zap1) and promoted by glucoamylases (Gca1 and Gca2), glucan transferases (Bgl2 and Phr1) and the exo-glucanase (Xog1). There are some evidences that β-(1,3)-glucan in biofilm protects *C. albicans* from neutrophil attacks (Mayer *et al.*, 2013).

7.5.3 Contact sensing and thigmotropism

Contact sensing is an environmental factor which initiates hyphae and biofilm formation. Yeasts change into hyphae by the contact with surface. Direct hyphal growth can occur by contact with surfaces with certain topology. This process is called thigmotropism and it is regulated by extracellular calcium uptake through calcium channels (Mayer *et al.*, 2013).

7.6 Extracellular hydrolytic enzymes

There are three most important families of extracellular hydrolytic enzymes: secreted aspartyl proteinases (Saps), phospholipase B enzymes (Plbs) and lipases (Lips). Proteomic analysis shows that *C. albicans* is able to secrete different types of proteins to adapt to the environment (Gil-Bona *et al.*, 2015b). These kinds of enzymes are important virulence factors. The virulence factors are components that cause damages to the host cell. The role of some of these enzymes is still under discussion.

7.6.1 Secreted aspartyl proteinases

Most of studies are focused on Sap family that is composed of ten members. While Sap1 to Sap8 are secreted into extracellular matrix, Sap9 and Sap10 are preserved at the cell surface by a GPI-anchor. Sap1–3 are required for mucosal infections, Sap4–6 for systemic infections. Sap9 and 10 are determinative for the infection process. However, their contribution to pathogenicity is still controversial (Schaller *et al.*, 2005, Albrecht *et al.*, 2006). They are connected with nutrient uptake, degradation of proteins and proposed to facilitate active penetration into host cells (Mayer *et al.*, 2013).

The most secreted Sap enzyme in media where proteins are the only source of nutrients is Sap2 (Figure 7). Saps enzymes are translated into preproenzymes on endoplasmic reticulum (ER) and directed by the *N*-terminal signal peptide into the ER to be secreted through the ER-to-Golgi or classical secretory pathway. The *N*-terminal sequence is removed in ER. Besides that, saps are processed by Kex2 (kexin) proteinase. After activation, Saps are transferred into vesicles and released into extracellular space. Sap9 and 10 are also secreted into vesicles that fuse with the cell membrane and where they stay anchored by a GPI group. There are more *Candida* species that are able to secrete Saps (Naglik *et al.*, 2003).

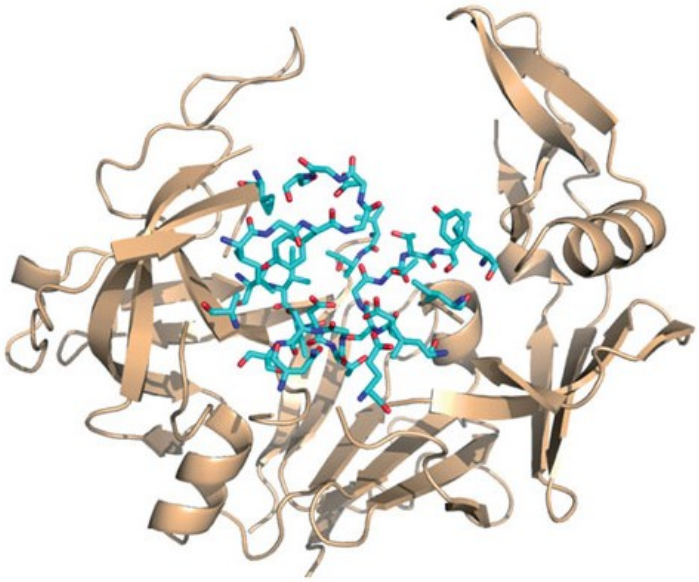


Figure 7: The molecular ribbon-like structure of Sap2. Major aspartyl proteinase in *C. albicans*.

Adapted from (Cassone, 2013).

7.6.2 Phospholipases

Phospholipases are enzymes responsible for hydrolysing the ester bonds in glycerophospholipids (Ghannoum, 2000). They are able to damage host cell membranes (Rossoni *et al.*, 2013). There are four classes of phospholipases family A, B, C and D. Only the five members of class B (Plb1–5) are extracellular. *C. albicans plb1Δ/Δ* and *plb5Δ/Δ* mutants are weakened in virulence in a mouse model of systemic infection. Their role is to contribute with disruption and evasion of tissue that is important in pathogenicity (Niewerth *et al.*, 2001).

7.6.3 Lipases

Third family is consisted of lipases. It has ten members Lip1–10 and all of them are important for pathogenicity (Stehr *et al.*, 2004). They probably play a role in adhesion and interaction with the immune system. This group of enzymes allows fat to be a source of carbon for pathogen (Hube *et al.*, 2000). Expression of their genes depends on the stage of infection. *N*-terminal sequence belongs to all Lips genes excepting Lip7. Majority of Lips (Lip1, 3, 4, 5, 6 and 8) are also expressed in media with lack of their substrates. Yeast-to-hyphal transition is the phase with the transcription of most lipases (Stehr *et al.*, 2004).

7.7 Adaptation to enviroment

Candida albicans is exposed to wide range of pH in human body. From slightly alkaline (pH 7.4) in blood stream and tissue to acidic in vagina (pH 4). The pH ranges approximately from two to eight in the digestive system. Thus, the adaptation is essential for survival. Neutral and alkaline pH causes huge stress to *Candida albicans.* In addition, there are changes of pH-sensitive proteins. Cell wall β-glycosidases Phr1 and Phr2 are important for the adaptation. Phr1 for neutral and alkaline pH (systemic infections) and Phr2 mainly in acidic environment (vagina).

Metabolic adaptation is very important for pathogenic *C. albicans* during infection. The main source of nutrients for *C. albicans* is glucose. Blood stream is quite rich in glucose concentration. However, *Candida* inside phagocytes suffers from starvation, thus activates gluconeogenesis and lipids and amino acids become sources of nutrients. The same situation happens in glucose-poor organs where proteins, amino acids lipids and phospholipids serve as a source of nutrients. There is a sufficient concentration of glucose in some organs and tissues like brain and liver. Yeasts can use secreted proteases to hydrolyse host proteins (Mayer *et al.*, 2013).

7.8 Secretory pathways

7.8.1 The classical secretory pathway

Secretion of yeasts is also important for adaptation to environment, protection from host defences, invading the host through secreted hydrolases. Secretion of proteins includes several organelles and other molecules (Schekman, 2010). It is a complex process which assures communication between cells and environment (Oliveira *et al.*, 2010). Secreted proteins are important not only for pathogenicity but also as a biomarker of infection and for potential acellular vaccines (Gil-Bona *et al.*, 2015b).

All eukaryotic cells are able to secrete lipids, proteins, polysaccharides and other types of macromolecules. Proteins with *N*-terminal signal sequence are designed to be secreted by classical secretory pathway (Shuster, 1991). This is the best known way of protein secretion also in yeasts, in which proteins travel through the ER, the Golgi apparatus and intracellular vesicles (Oliveira *et al.*, 2010). First part of secretion pathway is a transport through membrane of ER. This process is named translocation. There are

many reactions in ER like *O*-glycosylation, *N*-glycosylation, GPI-anchor addition and disulphide bond formation.

Maturation of proteins is performed by signal peptidase complex (SPC). It is a complex that removes the *N*-amino terminal signal sequence of secretory proteins. They are moved from ER into Golgi apparatus. Functional components of ER are contained in vesicles and they must be recycled from Golgi back into ER. It is called retrograde Golgi-ER transport (Fonzi W. A., 2008). Transport vesicles containing the secretory proteins are formed from Golgi complex and fuse with plasma membrane. The content of vesicles goes out of cell by the process called exocytosis (Oliveira *et al.*, 2010) (Figure 8).

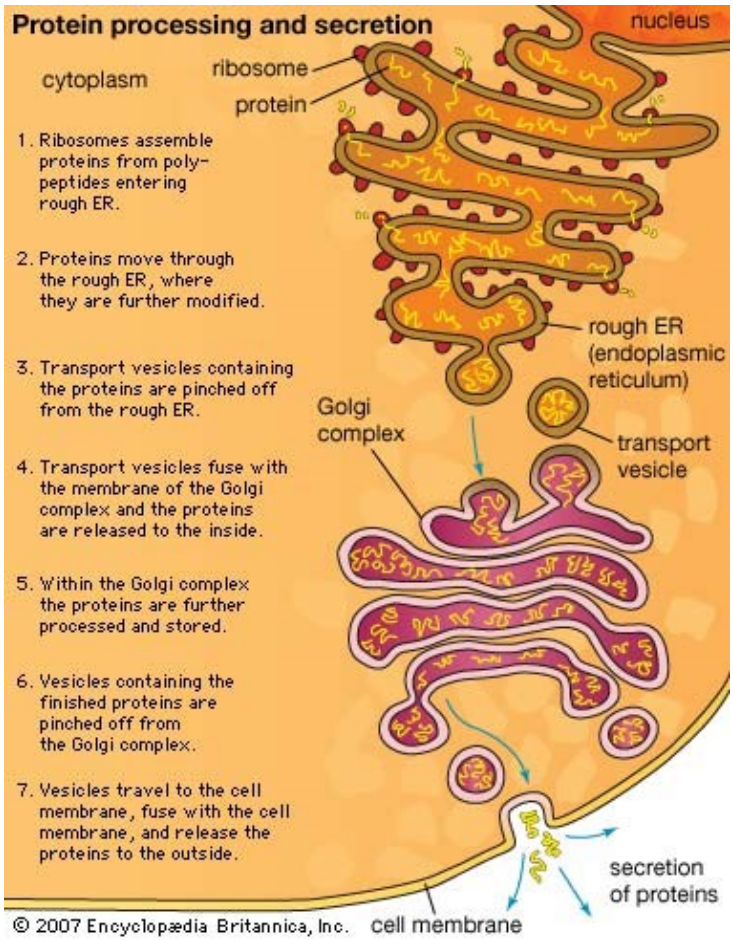


Figure 8: Classical secretory pathway.

Adapted from (Web 5 – http://kids.britannica.com/comptons/art-144468/The-endoplasmic-reticulum-plays-a-major-role-in-the-biosynthesis).

In addition to hydrolytic enzymes, there are more proteins secreted (for example the endoglucanase Eng1) in *C. albicans*. Also, there are 7 cell wall polysaccharide-processing enzymes secreted under various growth conditions (Cht3, Mp65, Scw11,

Sim1, Sun41, Tos1 and Xog1) and proteins responsible for separation of metal ions (such as zinc Pra1 and Zrt1 or iron Csa1, Csa2, Pga7, Pga10 and Rbt5) (Sorgo *et al.*, 2013).

7.8.2 Unconventional secretory pathways

Some proteins are sometimes present in media although they do not have *N*-terminal signal peptide (cytosolic proteins). That is the evidence for unconventional secretion. Several studies have detected this type of proteins at the cell wall or outside the cell and some unconventional secretory have been proposed in *C. albicans* (Nombela 2006).

Among unconventional secretory pathways belongs secretion by extracellular vesicles (EVs) (Oliveira *et al.*, 2010). This process is typical in eukaryotic cells but in fungi is unique because the yeasts possess a dynamic cell wall. Observation using transmission electron microscope (TEM) showed different sizes and types of extracellular vesicles in the culture media. However, the way of vesicle production is still unknown as well as passing through the plasma membrane and cell wall (Albuquerque *et al.*, 2008).

EVs are important structures for transporting virulence factors through the cell wall. Modulation of host immune cells is also performed by extracellular vesicles (Vallejo *et al.*, 2012). They serve to raise the pathogenicity of fungi (Rodrigues *et al.*, 2013). There are some evidences which confirm the hypothesis that EVs are released during infection (Rodrigues *et al.*, 2015).

Protein composition of EVs in *C. albicans* is similar to the other fungal species. They include cytoplasmic, mitochondrial, vacuolar, cell wall architecture components (sterols and glucosylceramide), plasma membrane, signalling and virulence related proteins (Oliveira *et al.*, 2010). Mainly proteomic analyses bring more complex characterisation of EVs in fungi. *Saccharomyces cerevisiae* serves as a model organism for EVs studies (Rodrigues *et al.*, 2013).

In fungi, there are also multivesicular bodies (MVBs) made up from invagination of plasma membrane as endosomes with production of internal vesicles and their releasing to extracellular environment as exosomes (a type of EVs) (Oliveira *et al.*, 2010). Essential mediator for MVBs biogenesis is the endosomal sorting complex required for transport (ESCRT) pathway (Rodrigues *et al.*, 2015). There are some evidences that secretion by exosomes includes elements from post-Golgi secretory pathway (Oliveira *et al.*, 2010). Another type of unconventional secretion connects MVBs and double-

membrane structure autophagosomes (secondary lysosome in which elements of a cell's own cytoplasm are digested), which fuse together and create amphisomes. They fuse with plasma membrane and release proteins and exosomes (Ding *et al.*, 2012). These processes are conserved between mammalian and fungi (a schematic representation of these processes in mammalian cells is showed in Figure 8).

Lots of differences have been shown in secretion of *C. albicans* and *S. cerevisiae.* Ability to cause diseases is closely connected with secretion. Virulence factors such as secretion of hydrolytic enzymes, phenotypic switching, yeast to hyphae transition, expression of cell surface adhesins, are bound to secretory pathways. There are over 200 genes included in transport process from intracellular to extracellular environment. Organisation of secretory apparatus differs in yeast and hyphae. Golgi is freely distributed through the cytoplasm in yeast however in distal part in hyphae. Golgi localisation does not require microtubules (Fonzi, 2009).

8 EXPERIMENTAL PART

8.1 Used material

8.1.1 Biological material

Tested strains: *C. albicans* wild type SC5314 (Gillum *et al.*, 1984) and RML2U (*ecm33Δ*) (Martinez-Lopez *et al.*, 2006), *C. albicans* cell wall and secretory mutants from Noble collection (Noble *et al.*, 2010).

8.1.2 Utilities

Sterile 96-well plate U bottom, parafilm, pipettes 1000 µL, 200 µL, 5–20 µL, sterile and non-sterile pipette tips 1000 µL, 200 µL and 10 µL, Petri dishes, inoculating loop, Eppendorf tubes 1.5 mL, Erlenmeyer flasks 100 mL

8.1.3 Instrumentation

Vortex Mixer - Heidolph Reax Top (Heidolph, Germany)

Centrifuge for Eppendorfs - Thermoscientific Heraeus Fresco 21 (Thermo Fisher Scientific, Massachusetts, USA)

Electrophoresis - Vertical Electrophoresis for SDS Page gels (Bio-Rad, California, USA)

Western Blot - Odyssey, Image Studio Lite (LI-COR, USA)

Incubator - Unitron HT Infors (Infors AG, Switzerland)

Autoclave - P Selecta Presoclave 75 (Kisker Biotech GmbH & Co. KG, Germany)

Spectrophotometer - Beckman DU 640 Spectrophotometer (GMI, Minnesota, USA)

Microplate Reader - Biorad Model 680 Plate Reader (Bio-Rad, California, USA)

8.1.4 Chemicals and media

DTT (dithiothreitol), loading buffer, Milli-Q water, TEMED (tetramethylethylenediamine), SDS 10% solution (sodium dodecyl sulphate), resolving and stacking buffer, acrylamide, PSA 10% solution (ammonium persulphate), PBS (phosphate-buffered saline), Coomassie blue stain, 0.5% Bromophenol Blue, Tris-HCl pH 6.8, D-glucose, ammonium sulphate, yeast nitrogen base (YNB), amino acids mix-ura, uracil, YPD (yeast extract peptone dextrose), BSA (bovine serum albumin), SD (synthetic

defined) liquid media (Table 1), YCB-BSA liquid media (Table 2), YPD solid media (Table 3).

Table 1: Composition of SD liquid media.

Substances	g/l
Glucose	20.00
Ammonium sulfate	5.00
Nitrogen base (YNB)	1.70
Amino acids mix-ura	1.92
Uracil	0.10

Table 2: Composition of YCB-BSA liquid media.

Substances	g/l
YCB	23.40
BSA	4.00

Table 3: Composition of YPD solid media.

Substances	g/l
Yeast extract	10.00
Peptone	20.00
Glucose	20.00
Agar	15.00

8.2 Methods

8.2.1 Transfer of *C. albicans* from glycerol onto YPD agar plate

Each *C. albicans* mutant was stored at -77°C and transferred from YPG (Yeast extract-peptone-glycerol in tubes) on the agar plates with YPD (yeast extract-peptone-

dextrose) solid media using sterile inoculating loops. Samples were incubated for 48 hours. This step was used for both methods (gel-based and 96-well plate protocol).

8.2.2 Gel-based protocol

This protocol is based on preculturing *C. albicans* wild types and mutants in SD liquid media overnight. Next step was their transfer into YCB-BSA liquid media with optical density of 0.2 and cultivation for 24 and 48 hours. This experiment was finished with SDS-PAGE electrophoresis and Coomassie blue staining. It was previously described by Staib and others (Staib *et al.*, 2008).

8.2.2.1 Preculturing in SD liquid media

SD medium was prepared. All substances were mixed and autoclaved at 121°C for twenty minutes. One colony was picked and transferred into 20 mL of liquid SD medium in 100 mL Erlenmeyer flasks by inoculating loop from each YPD solid plate. Samples were precultured at 30°C with continual shaking (200 rpm (revolutions per minute)) over night (O/N).

8.2.2.2 Transfer from SD into YCB-BSA liquid media

YCB-BSA medium was prepared. YCB was dissolved in deionized water (dH_2O). The pH was adjusted to 4.0 with diluted hydrochloric acid and subsequently autoclaved at 121°C for 20 minutes. BSA was dissolved in a small amount of dH_2O and pH was also adjusted to 4.0. When the temperature of autoclaved solution of YCB was lower than 55°C, the BSA solution was filtered making use of bacterial filter (0.22 μm) into it. It was necessary to mix it well.

The diluted samples (1:10) were made up (100 μL from flask with mutant sample in SD liquid media was taken and resuspended in 900 μL of pure SD liquid media in the Eppendorf tube). 1 mL of SD media was used as a blank. Absorbance was measured in order to count initial volume of sample to prepare OD (optical density or absorbance) of 0.20 in 20 mL of YCB-BSA liquid media. The dilution 1:10 is necessary for the measurement of OD of *C. albicans* at a λ of 600 nm.

8.2.2.3 Collection of supernatant

1 mL of sample in YCB-BSA liquid media was used for collecting supernatant after 24 and 48 hours of cultivation. Collecting was performed by a centrifugation for ten

minutes at 10 000 rpm, the cell pellet was saved for western blot and the supernatant was used for gel study. The supernatant was stored in the freezer at -20°C.

8.2.2.4 SDS-PAGE electrophoresis

For SDS-PAGE electrophoresis was necessary to prepare:

- SDS-PAGE gel (Table 4):

Table 4: Composition of SDS-PAGE gel 12%.

COMPOSITION	RESOLVING gel		STACKING gel	
	1	2	1	2
dH_2O (deionised)	2.6 mL	5.3 mL	2.6 mL	5.3 mL
Tris-HCl (1M pH 8.8)	2 mL	4 mL	–	–
Tris-HCl (1M pH 6.8)	–	–	1.25 mL	2.5 mL
Acrylamide (30%)	3.2 mL	6.4 mL	1 mL	2 mL
SDS (10%)	80 µL	160 µL	50 µL	100 µL
PSA (10%)	80 µL	160 µL	50 µL	100 µL
TEMED	8 µL	16 µL	5 µL	10 µL
12%				

It is necessary to use the solutions before it starts to solidify. The solution was put into space between two glass plates in a casting frame stabilized in casting stand. The electrophoretic comb was put between these two glass plates immediately before the solutions started to solidify. The comb serves as an aid for making sample wells.

- Samples for electrophoresis:

10 µL of supernatant, 10 µL of loading buffer five times concentrated (250 mM Tris-HCl pH 6.8, 500 mM DTT, 10% SDS and 0.5% Bromophenol Blue) together with 1 µL of DTT were put into the Eppendorf tube. The Eppendorf tubes were put into 99°C for five minutes in order to denature proteins. The samples had to be stored in the ice.

The gel between glassy frames was put into gel box. The gel box was filled with running buffer (25 mM Tris base, 190 mM glycine, 0.1% SDS) pH 8.3 and the comb was removed and checked if the wells are good quality. The wells were filled with 3 µL of protein marker, 18 µL of each sample or 18 µL of positive control that was liquid YCB-BSA medium. The electrophoresis ran for 1 hour ± 15 minutes with voltage 100 V until the line of samples was at the bottom of the gel (Figure 10). Gel was removed from glassy frames and coloured with Coomassie blue (one hour). For decolourization, a

solution of 40% methanol and 10% acetic acid was used for another one hour. The water was used to keep the gel wet after decolourizing.

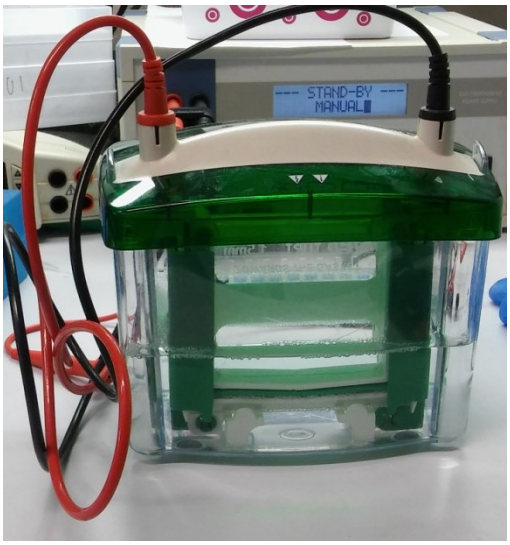


Figure 9: SDS-PAGE electrophoresis.

Author: Nikola Kollárová Universidad Cumplutense Madrid, Faculty of Pharmacy, Department of Microbiology II.

8.2.3 96-well plate analysis

8.2.3.1 Optimisation of the method

The protocol with 96-well plates was designed in order to detect the degradation of BSA in YCB-BSA liquid media. It was based on the correlation between OD_{600} and the presence of a band (corresponding with BSA) in SDS-PAGE gel. The reason for using 96-well plate was to gain a simple, fast method with possibility to test bigger amount of samples and use lower amounts of culture media. First of all, we make some preliminary experiment looking for the best conditions to make the experiment. It was necessary to use plates with U-bottom to see compact pellets (Figure 11). It was not necessary to take the previous step of the overnight preculturing in SD liquid media (used in the gel-based protocol) so it saved one day of experiment. Also, the resuspension of the colony could be completed in sterilized water, not in the media. The size of the taken colony was not decisive to the final OD obtained. There was observed evaporation in the outer wells of 96-well plates during incubation at 30°C that could be easily eliminated sealing the plate with parafilm. This did not affect the growth of *C. albicans*. It was observed that, it was better to incubate without shaking to prevent cross-contamination. It was also very

important to optimize the time for collecting the data. At 24 hours neither wild type (SC5314) grew sufficiently. Based on several experiments with 96-well plates, we found that the best conditions for cultivation were 48 hours at 30°C without shaking sealed with parafilm.

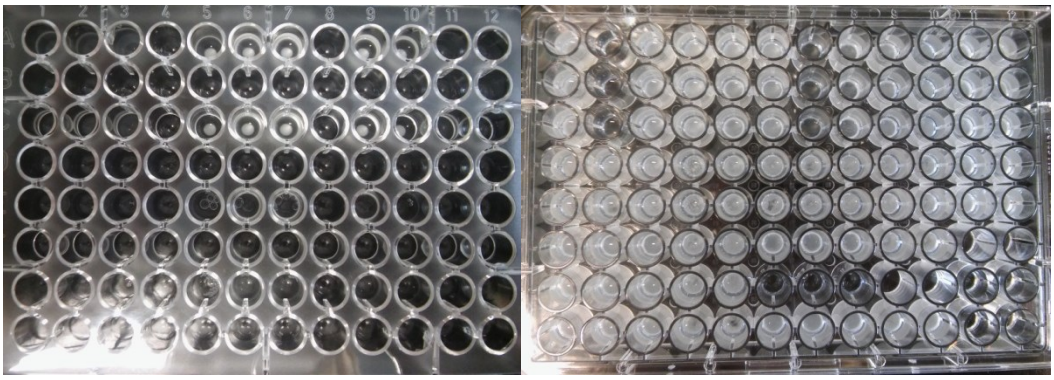


Figure 10: Difference of the pellets in 96-well plate U-bottom (left side) and 96-well plate flat bottom (right side).

Author: Nikola Kollárová, Universidad Complutense Madrid, Faculty of Pharmacy, Department of Microbiology II.

8.2.3.2 Protocol for 96-well plate

One colony from solid YPD plate was transferred and resuspended in 1 mL of sterile H_2O in Eppendorf tube. Wells were filled with SD and YCB-BSA liquid media: SD media to see if the growth is typical in that media and YCB-BSA media to see if they degrade BSA (bovine serum albumin). The blank for both media (SD and YCB-BSA) was required to see if sterile conditions were maintained during the experiment. Three replicates of each mutant in YCB-BSA media and two replicates of each mutant in SD media were used to see if there would be differences between both culture media and to allow statistical analyses. The OD_{600} was measured in the microplate spectrophotometer without shaking and cap. In that way, it was possible to analyze sixteen mutants in one 96-well plate plus the positive control for BSA-degradation (the wild type SC5314) and as a negative control for non-BSA degradation (RML2U mutant).

8.2.3.3 Statistical analysis

The average values from three replicates for each mutant and wild type were counted and paired t-test statistics performed in Graphpad program. The principle of this method is to determine if two sets of data are significantly different from each other. Program gives “p” values and tells if the differences between these two conditions

(growing in SD and YCB-BSA liquid media) are significant (p value is <0.01 (**), <0.05 (*)) or if not (p>0.05).

8.2.4 Protein extraction

Preparation of protein extracts from culture supernatant and cytoplasmic extract for SDS-PAGE and western blot (WB):

8.2.4.1 Growth conditions

1. One colony of *C. albicans* from YPD solid media was transferred into 20 mL of SD liquid media and cultivated for 7 hours at 30°C and 200 rpm.
2. Counted amount of the preculture into 20 mL of SD liquid media in order to obtain OD_{600} of 4.00 after growing at 30ºC and 200 rpm 16 hours later.
3. Whole amount was centrifuged and supernatant and pellet were collected.

8.2.4.2 *C. albicans* cytoplasmic protein extract

1. Pellet was washed twice with PBS.
2. Than resuspended in 1 mL of the lysis buffer (50 mM Tris-HCl pH 7.5, 1 mM EDTA, 150 mM NaCl) and into 1 mL of this add 1 µL DTT + 10 µL protease inhibitors, 5 µL of phenylmethanesulfonylfluoride (PMSF).
3. Equal volume of 0.5–0.75 mm diameter glass beads was added.
4. Cells were disrupted in a Fastprep cell breaker (five cycles of 30 seconds for each one), between each breaking cycle let samples for five minutes in the ice.
5. Cell extract was separated from glass beads and cell debris 1100 rpm for one minute.
6. It was clarified at 13000 rpm, for 30 minutes.
7. Supernatant was collected and frozen at -80°C.

8.2.4.3 Bradford method

The Bradford protein assay was used to measure the concentration of protein in a sample. The principle of this assay is that the binding of protein molecules to Coomassie dye under acidic conditions results in a color change from brown to blue. This method actually measures the presence of the basic amino acid residues, arginine, lysine and histidine, which contributes to the formation of the protein-dye complex.

PROTOCOL:

1. Standard calibration curve (seven tubes with different dilutions of BSA) and the samples were prepared (Table 5).

Table 5: Bradford assay-dilutions of samples for calibration curve and composition of sample for Bradford method.

Tube	1	2	3	4	5	6	7	sample
BSA (2mg/mL) (µL)	0.0	0.5	1.0	2.0	4.0	8.0	16.0	5.0
Amount of proteins (µg)	0	1	2	4	8	16	32	–
Bradford reagent (µL)	200	200	200	200	200	200	200	200
dH_2O (µL)	800.0	799.5	799.0	798.0	796.0	792.0	784.0	795.0

2. Samples were vortexed and incubated in a room temperature for 20 minutes.
3. The absorbance was measured at 595 nm.
4. The calibration curve was made up and the results were interpolated.

8.2.4.4 Western blot

This method was performed to compare presence of Sap2 in supernatant and cytoplasm.

PROTOCOL:

1. SDS-PAGE electrophoresis gel was prepared.
2. Samples:
   a) 10 µL of supernatant + 1 µL of DTT + 10 µL of loading buffer.
   b) Counted amount of cytoplasmic extract + counted amount of Milli-Q H_2O + 1 µL of DTT + 7.3 µL of loading buffer.
3. Electrophoresis.
4. The proteins were transferred from the gel to the nitrocellulose membrane in a transfer tank filled with transfer buffer (25 mM Tris base, 180 mM glycine, 20% methanol) pH 8.3, for 1 hour at 100 V (Figure 12).
5. The nitrocellulose membrane was put into red Ponceau for ten minutes.
6. Washed with PBS.
7. Blocked for one hour with 5% milk at room temperature.

8. The membrane was incubated with a primary antibody solution (Sap2 antibody was a gift from Dr. M. Monod, Centre Hospitalier Universitaire Vaudois, Switzerland) at 1:3000) overnight at 4°C.
9. Washed with PBS (1x) tween.
10. The membrane was incubated with the dilution 1:10000 (PBS tween 1% milk) of conjugated secondary 1/2000 IRDye 800 goat antirabbit IgG (LI-COR Biosciences) in blocking buffer at room temperature for one hour.
11. Washed three times for five minutes with PBS.
12. Scanned by Odyssey® CLx Imaging System.

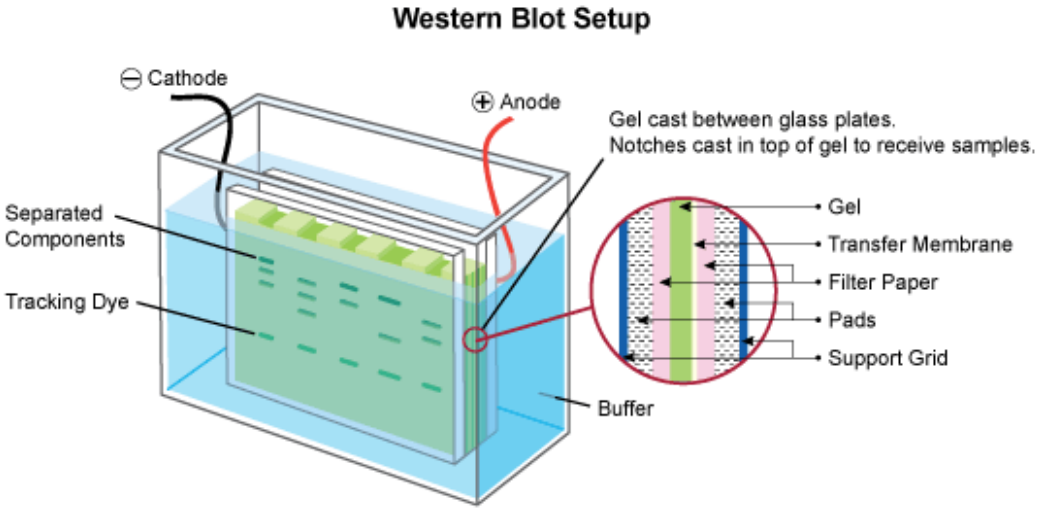


Figure 11: Western blot setup**.**
Adapted from (Web 6 – http://www.leinco.com/general_wb).

8.2.5 Databases and bioinformatic tools

Candida genome database (CGD) was used to find *C. albicans* cell-wall and secretory mutants involved in these processes (transport, vesicle-mediated transport, protein folding and organelle organization) (Skrzypek M. S. *et al.*, 2017, http://www.candidagenome.org/cgi-bin/GO/goTermMapper). The mutants were sorted by online available Venny software into few groups showing how many and which mutants are included in selected process or more processes (Figure 13) (Oliveros J. C. 2007–2015, http://bioinfogp.cnb.csic.es/tools/venny/). String program shows relationship between selected mutants in protein homologues in *Saccharomyces cerevisiae* (Figure 14)

(http://string-db.org/cgi/input.pl?UserId=6l6ovGJN106X&sessionId=Ph0HF906cSNp&input_page_active_form=multiple_identifiers).

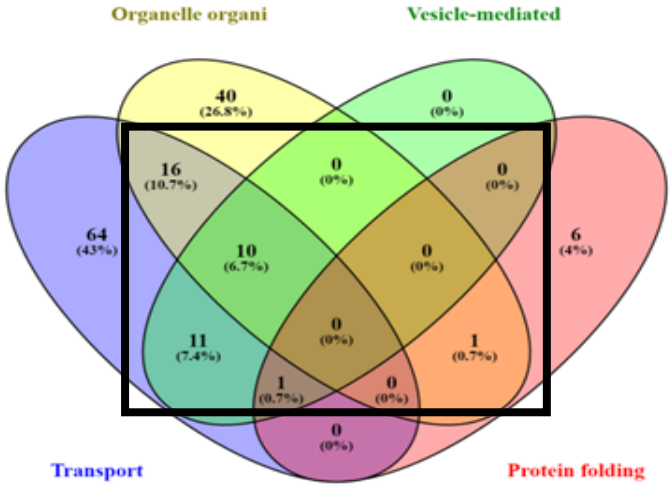


Figure 12: Number of mutants involved in certain process or processes (organelle organization – yellow; vesicle-mediated transport – green; transport – purple and protein folding – pink). Data in square: tested mutants.

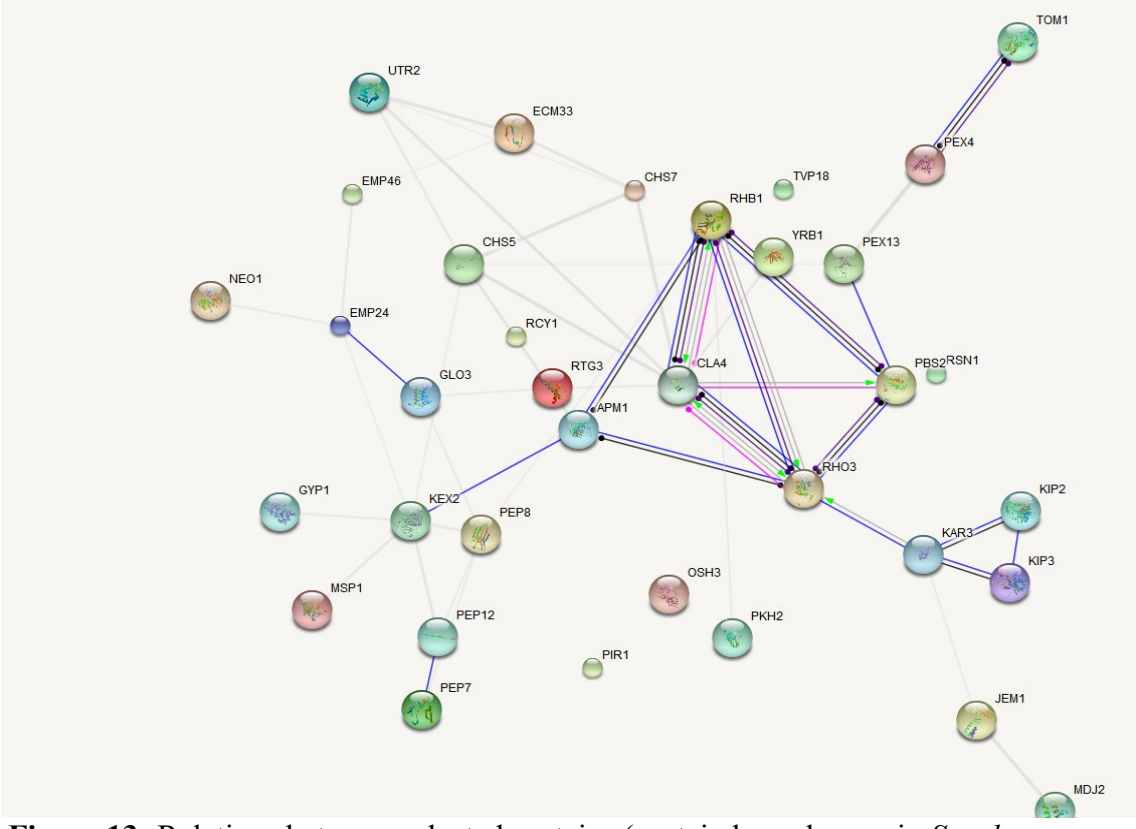


Figure 13: Relations between selected proteins (protein homologues in *Saccharomyces cerevisiae*).

Data adapted from www.string-db.org.

9 RESULTS

9.1 Mutant selection

To look for *C. albicans* proteins involved in Sap2 secretion, we made different experiments. Secretion of Sap2 in mutants was detected by absence of BSA band (66.5 kDa) in Coomassie blue stained gel after 24 and 48 hours of growth in YCB-BSA liquid media. In this medium, BSA is the sole source of nitrogen. It is degraded mainly by Sap2 protein, therefore mutants that cannot secrete this protein were not able to grow in this medium and subsequently die. That was the reason of correlation between OD and results in the SDS-PAGE gel. This fact was used for optimising new method using 96-well plates. The aim was to obtain a faster, easier and miniaturized method with lower amount of media and sample needed.

The selection of mutants in a first set was based on previous preliminary results with mutants of certain proteins related with cell-wall structure (*ecm33Δ*) or related with glucan metabolism (*pir1Δ*), chitin metabolism (*chs5Δ*) and crosslinking (*utr2Δ*) or protein secretion (*ypt72Δ, kex2Δ*) in *C. albicans*. In the second set other interesting mutants connected with protein secretion specifically involved in transport (*e.g. apm1Δ, emp46Δ, pep8Δ, tom1Δ, rho3Δ, cst20Δ, cla4Δ, pbs2Δ*), organelle organisation (*e.g. apm1Δ, tom1Δ, rho3Δ, cst20Δ, cla4Δ, pbs2Δ*) and vesicle-mediated transport (*e.g. apm1Δ*, *emp46Δ*, *pep8Δ*) were chosen (selected by Goterm enrichment process at CGD).

Total number of mutants correlated with combination of four selected processes (transport, vesicle-mediated transport, protein folding and organelle organization) was 39 and they include 2 from the previously 6 selected (*ecm33Δ* and *kex2Δ*) (Table 6). Therefore, 43 mutants were selected to be analyzed. As there were found 3 mutants that did not grow due to glycerol in YPD solid media (*kip*3Δ, *kin2Δ* and *arl1Δ*), the total number of analyzed mutants was 40.

Table 6: List of processes and *C. albicans* mutants involved in these processes. Data adapted from www.candidagenomedatabase.org.

PROCESSES	STRAINS
TRANSPORT + VESICLE-MEDIATED TRANSPORT + PROTEIN FOLDING	*chs7Δ*
ORGANELLE ORGANISATION + PROTEIN FOLDING	*jem1Δ*
TRANSPORT + ORGANELLE ORGANISATION + VESICLE-MEDIATED TRANSPORT	*pep12Δ, apm1Δ, neo1Δ, orf19.1567Δ, emp24Δ, ypt72Δ, osh3Δ, pep7Δ, kin2Δ, glo3Δ*
TRANSPORT + VESICLE-MEDIATED TRANSPORT	*rhb1Δ, arl1Δ, chs5Δ, emp46Δ, rsn1Δ, pep8Δ, gyp1Δ, orf19.433Δ, tvp18Δ, rcy1Δ, orf19.3763Δ*
TRANSPORT + ORGANELLE ORGANISATOIN	*pex13Δ, pkh2Δ, orf19.6980Δ, tom1Δ, rho3Δ, pbs2Δ, cla4Δ, kar3Δ, kip2Δ, rtg3Δ, msp1Δ, cag1Δ, kip3Δ, mdj2Δ, cst20Δ, pex4Δ*

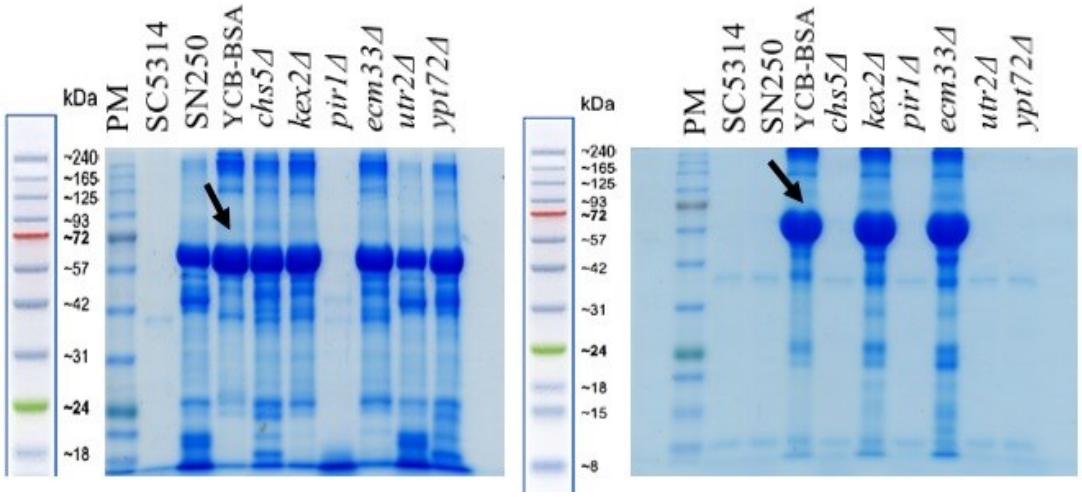
9.2 Detection of Sap2 in supernatant: gel-based protocol

9.2.1 Analysis of the first set of mutants

We analysed the Sap2 secretion of the six mutants indicated in Figure 14 and Table 8 using the method described by Staib and others (Staib *et al.,* 2008) that is based on the observation that Sap2 non-secreting mutants are unable to degrade BSA and this can be checked by SDS-PAGE in this first analysis. Two wild type strains (SC5314 and SN250) were included in the analysis as controls (they are able to degrade BSA). The *ecm33Δ* mutant was chosen as the control of non-degradation of BSA because this result was previously obtained in the Microbiology II Department at UCM (Gil-Bona *et al.*, 2015b).

Cultures of the strain in YCB-BSA were prepared and 10 µL of the supernatant was analysed in a gel after 24 or 48 hours of incubation. In addition, 10 µL of YCB-BSA were added as a control of BSA that can be easily observed (66 kDa) (Figure 15).

In 24 h samples, only wild type SC5314 and mutant *pir1Δ* degraded BSA. The other wild type (SN250) and *utr2Δ* seemed to start degrading BSA. The *chs5Δ, kex2Δ*, *ecm33Δ* and *ypt72Δ* mutant strains did not degrade BSA at 24 h of growth. After 48 h, all wild types and mutants except for *kex2Δ* and *ecm33Δ*, were able to degrade BSA.



Note: PM-protein marker, numbers – molecular mass (kDa-kilodaltons), ↓-BSA.

Figure 14: SDS-PAGE electrophoresis and Coomassie blue staining of gel after 24 h (left side) and 48 h (right side) of cultivation in YCB-BSA liquid media.

The OD_{600} of the cultures at the 24 and 48 hours was also measured and it was observed that, as expected mutants with defects in Sap2 secretion or BSA degradation were also unable to grow in this media because BSA is the only nitrogen source. Thus we could conclude that the results correlated well with the measured OD_{600} (Table 7).

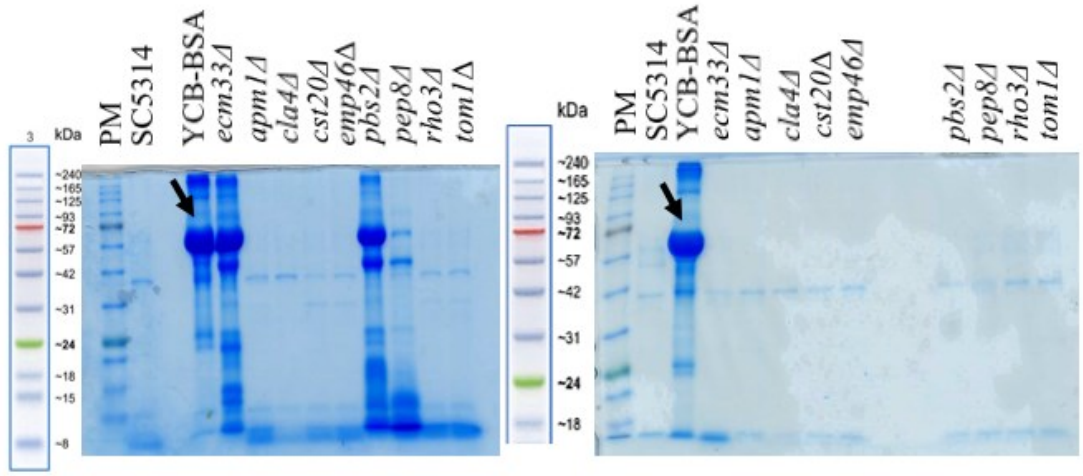
Table 7: OD of samples in SD liquid media (overnight) and YCB-BSA liquid media (after 24 and 48 hours).

Strains	OD_{600} overnight SD media	OD_{600} 24 hours YCB-BSA media	OD_{600} 48 hours YCB-BSA media
SC5314	4.60	8.60	10.41
SN250	5.19	1.40	10.70
***chs5*Δ**	5.80	**0.50**	9.19
***kex2*Δ**	4.82	**0.46**	**0.59**
*pir1*Δ	4.78	4.40	10.50
ecm33Δ	3.30	**0.28**	**0.83**
*utr2*Δ	5.36	1.70	10.70
***ypt72*Δ**	5.16	**0.94**	9.46

Note: Data in bold refer non-degradation of BSA.

9.2.2 Analysis of the second set of mutants.

In this second analysis we analysed the Sap2 secretion of the eight mutants indicated in Figure 16 and Table 8 using the method as in the analysis of the first set of mutants.



Note: PM – protein marker, numbers – molecular mass (kDa – kilodaltons), ↓-BSA.

Figure 15: SDS-PAGE electrophoresis and Coomassie blue staining of gel after 24 h (left side) and 48 h (right side) of cultivation in YCB-BSA liquid media.

Table 8: OD of samples in SD liquid media (overnight) and YCB-BSA liquid media (after 24 and 48 hours).

Strains	OD_{600} overnight SD media	OD_{600} 24 h YCB-BSA media	OD_{600} 48 h YCB-BSA media
SC5314	7.27	9.71	13.88
***ecm33*Δ**	6.87	**1.14**	11.51
*apm1*Δ	6.74	9.77	10.87
*emp46*Δ	8.33	11.21	13.09
*cla4*Δ	4.51	8.94	10.69
*cst20*Δ	8.10	10.29	13.10
***pbs2*Δ**	6.96	**3.17**	11.49
*pep8*Δ	6.52	5.43	12.41
*rho3*Δ	8.10	9.97	12.73
*tom1*Δ	7.99	9.91	13.16

Note: Data in bold refers to non-degradation of BSA.

The same correlation between OD_{600} and results in the gel was obtained in the analysis of this set of mutants, also using wild type as negative control and YCB-BSA and *ecm33*Δ as the positive controls. Only *pbs2*Δ mutant was not able to degrade BSA

after 24h. *Pep8*Δ mutant strain had started to degrade at this time. All mutants including *pbs2*Δ were able to degrade BSA after 48h.

These two experiments confirmed the relationships between results from SDS-PAGE gel and OD_{600}, showing the optimization of 96-well plate assay.

9.3 Detection of Sap2 in supernatant: miniaturised protocol

The Sap2 secretion of the 40 selected mutants (Table 6, Figures 17–23) which had grown in YPD solid media utilizing glycerol using the optimised method described in the chapter 8.2.3.1 was analysed in the miniaturised protocol with 96-well plate. It is based on the observation of BSA degradation in 96-well plates and this phenomenon can be checked by measurement of OD_{600} after 48 hours of cultivation. The time 48 hours was selected because the data were more reproducible. SD liquid media was used to test that mutants have not a general growth defect. The wild type strain (SC5314) was included in the analysis as control (it is able to degrade BSA) and the *ecm33Δ* mutant was the control of non-degradation of BSA as in gel-based protocol. The wild type SC5314 reaches ODs around 1.4 in YCB-BSA media and 1.0 in SD. The *ecm33Δ* 0.1 in YCB-BSA media and around 0.9 in SD.

Results from miniaturized protocol (in 96-well plate) are represented in the following graphics (Figures 17–23). Error bars indicate standard deviations of three independent replicates. The data indicates the average OD_{600} in three independent replicates for each mutant strain (the optical density is reduced by optical density of the blank of the growth media). Significant growing difference in SD and YCB-BSA media by t-test is described by (**) - p value is $p<0.01$ and (*) - p value is $0.01<p<0.05$. Three categories of mutants were found: mutants that grew similar to the wild type, mutants that showed significant differences between SD and YCB-BSA growth and mutants with an intermediate phenotype (they do not showed significant differences but grew less in YCB-BSA than in SD, opposite to wild type strains). The mutants of two last categories are listed in Table 9.

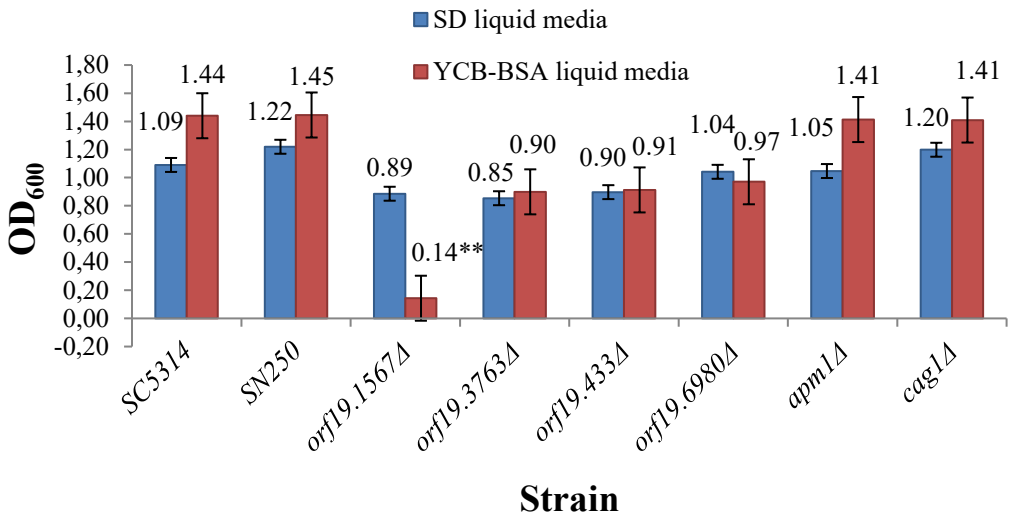


Figure 16: 48 hours average growth of two wild types (SC5314 and SN250) and mutants in SD and YCB-BSA liquid media in 96-well plate experiment.

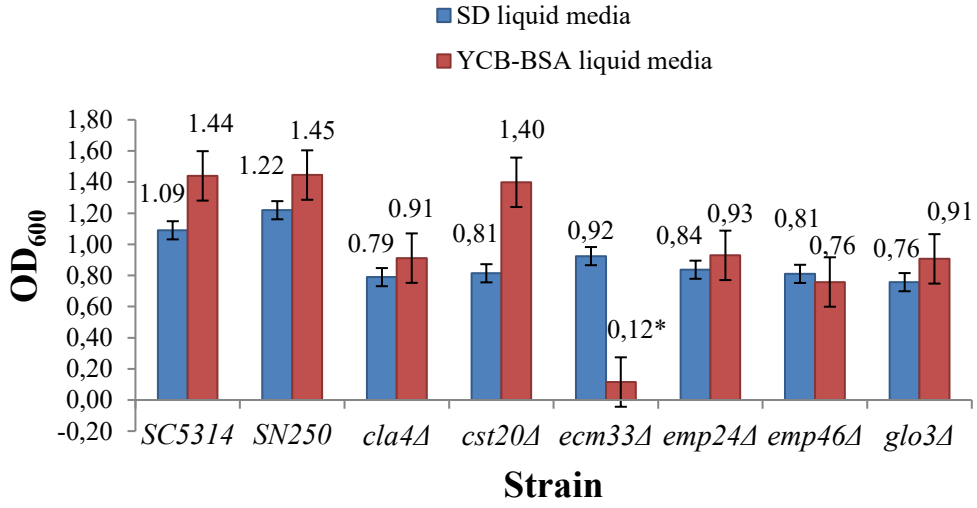


Figure 17: 48 hours average growth of two wild types (SC5314 and SN250) and mutants in SD and YCB-BSA liquid media in 96-well plate experiment

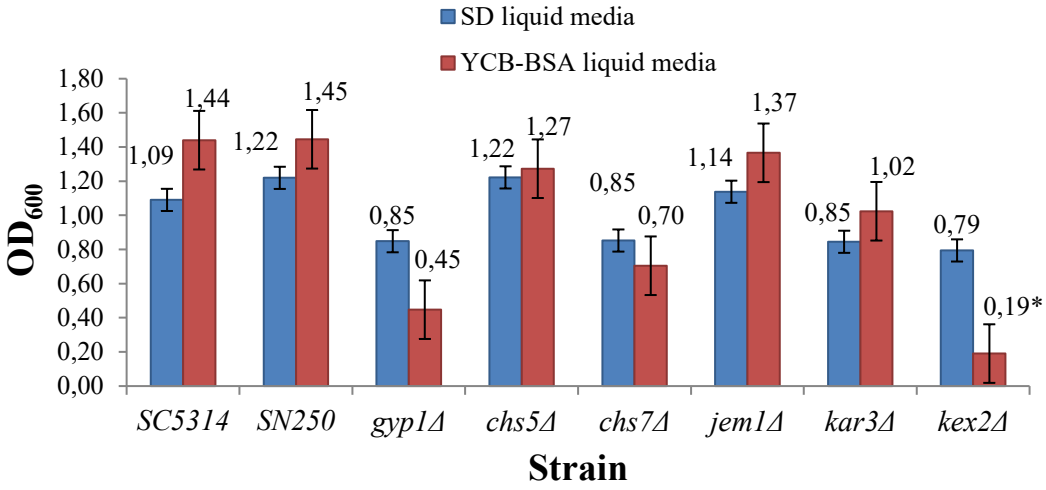


Figure 18: 48 hours average growth of two wild types (SC5314 and SN250) and mutants in SD and YCB-BSA liquid media in 96-well plate experiment.

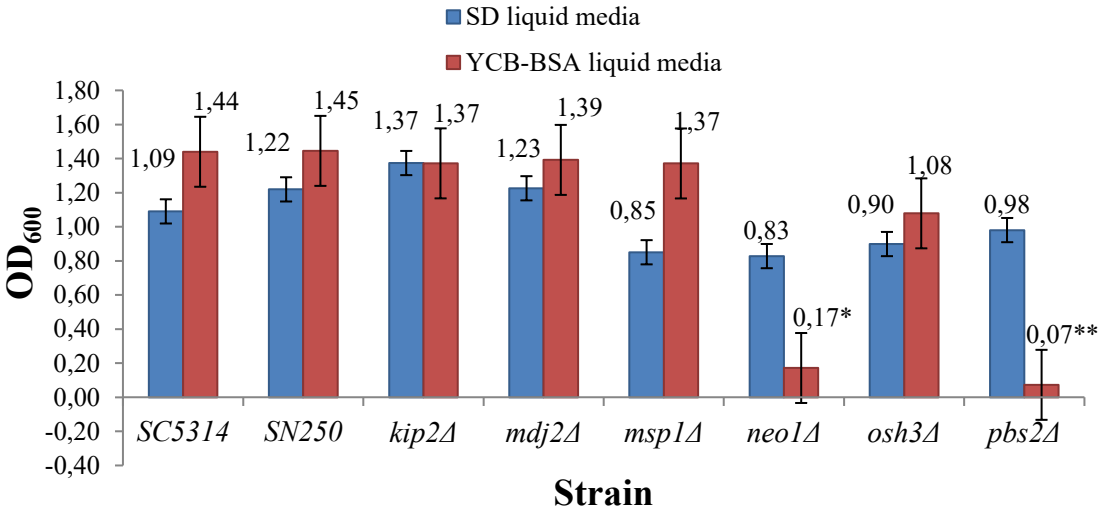


Figure 19: 48 hours average growth of two wild types (SC5314 and SN250) and mutants in SD and YCB-BSA liquid media in 96-well plate experiment.

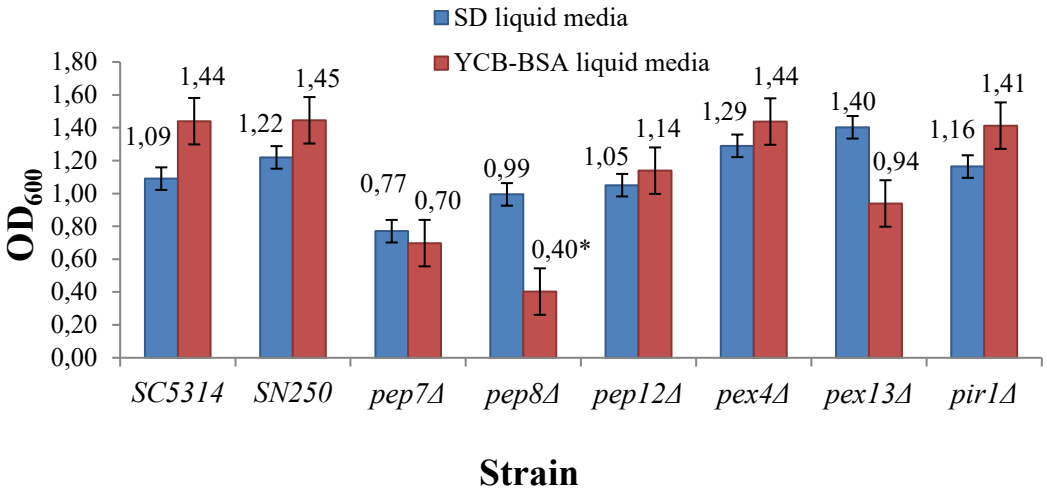


Figure 20: 48 hours average growth of two wild types (SC5314 and SN250) and mutants in SD and YCB-BSA liquid media in 96-well plate experiment.

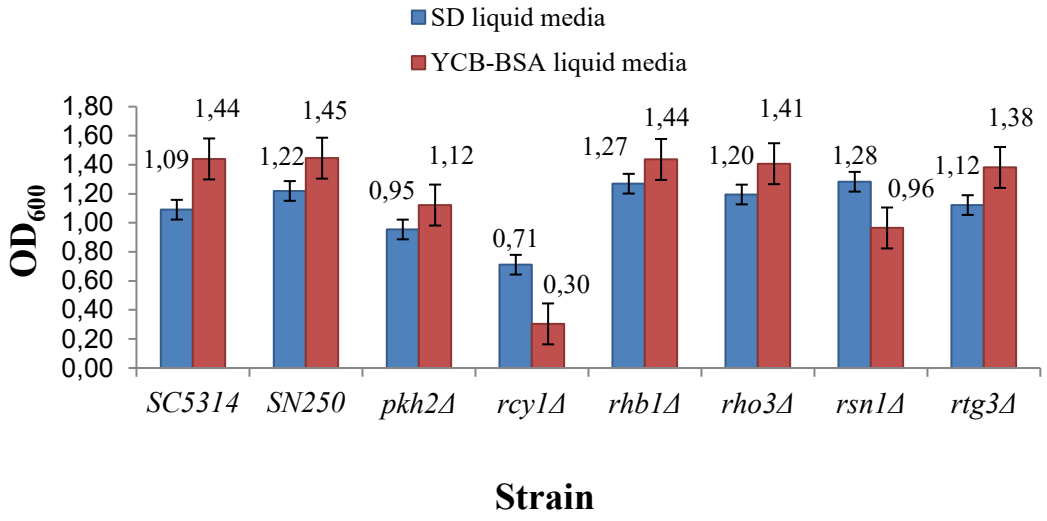


Figure 21: 48 hours average growth of two wild types (SC5314 and SN250) and mutants in SD and YCB-BSA liquid media in 96-well plate experiment.

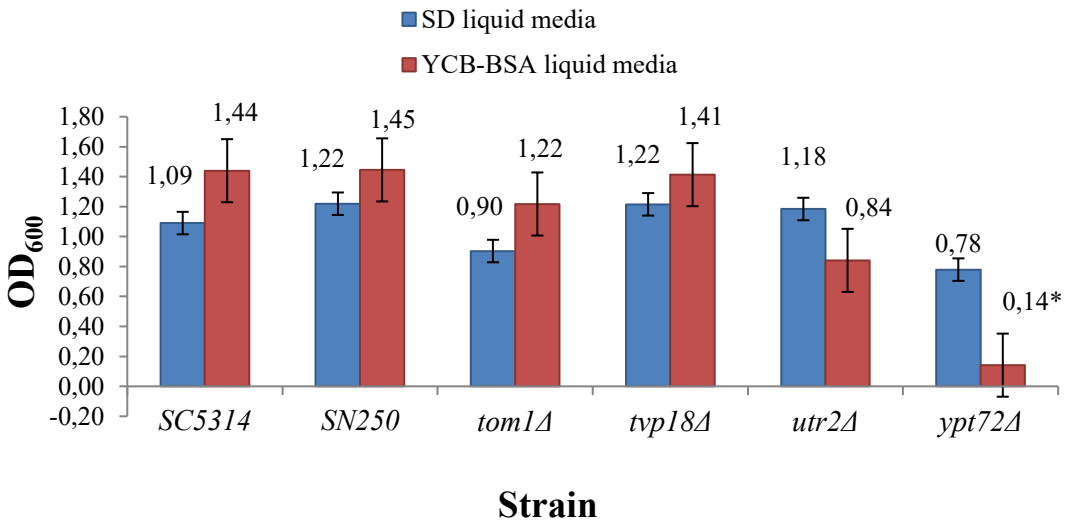


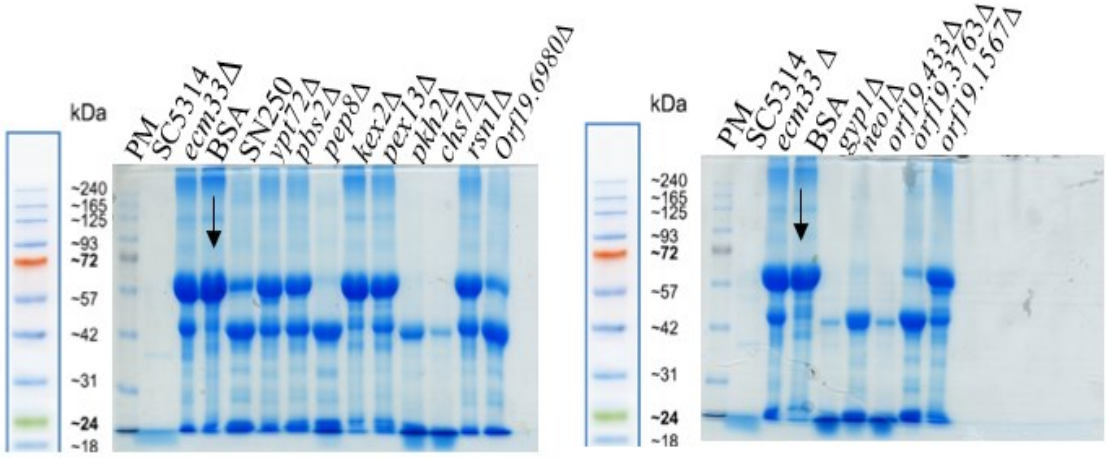
Figure 22: 48 hours average growth of two wild types (SC5314 and SN250) and mutants in SD and YCB-BSA liquid media in 96-well plate experiment.

Table 9: T-test results of mutants from 96-well plate protocol that grew different than the wild type strains.

Strains	P value	P<0.05	Role of the protein in *C. albicans*
ecm33Δ	0.0300	Yes	Ecm33 is involved in maintenance of cell wall integrity Kex2 is involved in Sap2 processing
kex2Δ	0.0139	Yes	
neo1Δ	0.0188	Yes	"Transport", "Organelle organization" and "Vesicle-mediated transport"
orf19.1567Δ	0.0039	Yes	
ypt72Δ	0.0019	Yes	
pep8Δ	0.0142	Yes	"Transport" and "Vesicle-mediated transport"
rcy1Δ	0.2223	No	
gyp1Δ	0.1834	No	
pbs2Δ	0.0011	Yes	"Transport" and "Organelle organisation"

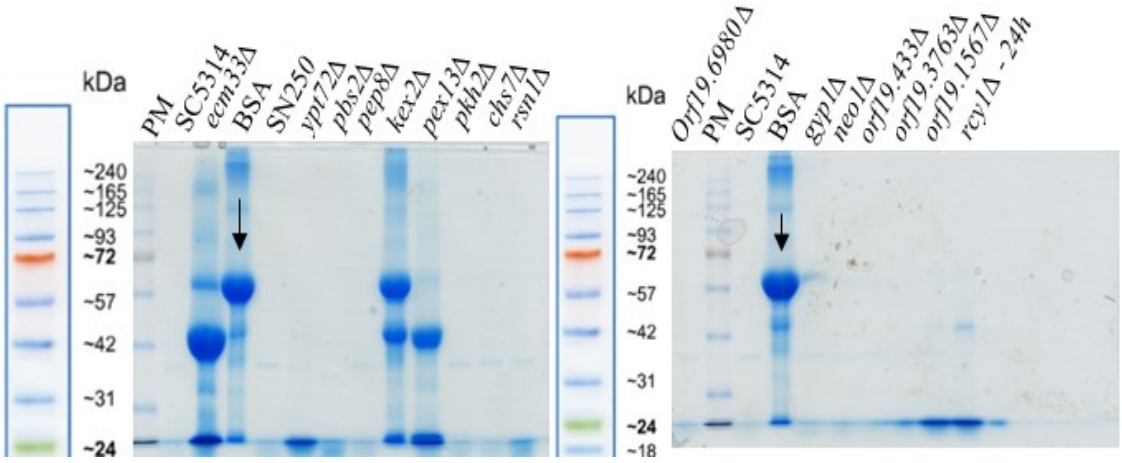
Note: (P<0.05) = statistical significance. Mutants with P<0.05 have significant difference in grow in SD and YCB-BSA liquid media.

The gel-based protocol was performed taking advantage of SDS-PAGE electrophoresis and Coomassie blue staining to confirm the gathered data. It was used for mutant strains, which did not degrade BSA at least in one replicate or there was intermediate result in 96-well plate experiment and the OD_{600} was measured (Figures 24, 25 and Table 10).



Note: PM – protein marker, numbers – molecular mass (kDa – kilodaltons), ↓-BSA.

Figure 23: SDS-PAGE electrophoresis and Coomassie blue staining of gel after 24 hours of cultivation in YCB-BSA liquid media with mutants, which at least in one replicate did not degrade BSA in 96-well plate.



Note: *Rcy1*Δ sample is from 24 hours of cultivation and 48 hours old sample is missing. PM – protein marker, numbers – molecular mass (kDa – kilodaltons), ↓-BSA.

Figure 24: SDS-PAGE electrophoresis and Coomassie blue staining of gel after 48 hours of cultivation in YCB-BSA liquid media with mutants, which at least in one replicate did not degrade BSA in 96-well plate.

Table 10: Optical densities of wild types and mutants in SD liquid media overnight, in YCB-BSA liquid media after 24 and 48 hours of cultivation.

Strains	OD_{600} overnight SD media	OD_{600} 24 hours YCB-BSA media	OD_{600} 48 hours YCB-BSA media
SC5314	7.06	9.13	10.66
SN250	7.52	0.59	10.35
***ecm33*Δ**	2.06	**0.43**	0.96
***kex2*Δ**	7.15	**0.50**	**0.76**
*pep8*Δ	4.95	0.59	9.61
***pbs2*Δ**	5.93	**0.56**	9.13
***pex13*Δ**	6.49	**0.51**	1.05
*pkh2*Δ	6.89	1.19	11.57
***rsn1*Δ**	7.30	**0.45**	9.92
*orf19.6980*Δ	6.81	0.53	12.06
*gyp1*Δ	6.19	2.54	10.74
*rcy1*Δ	0.69	3.22	NO
*neo1*Δ	4.47	0.84	9.79
*orf19.433*Δ	7.40	2.27	9.78
*orf19.3763*Δ	5.10	0.45	9.01
***orf19.1567*Δ**	6.09	**0.26**	3.83
*chs7*Δ	5.57	2.41	10.08
***ypt72*Δ**	5.75	**0.38**	4.78

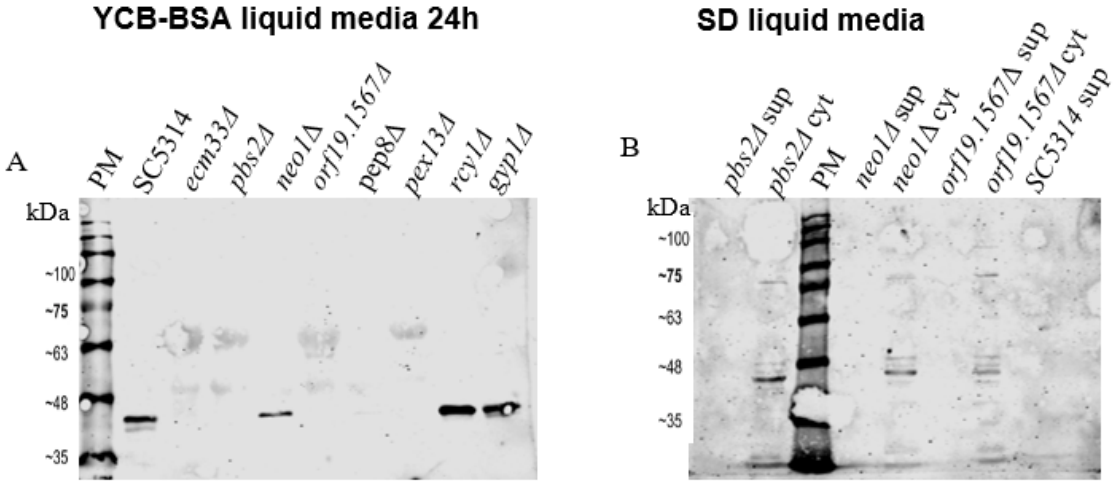
Note: Data in bold refer non-degradation of BSA.

RESULTS FROM SDS-PAGE ELECTROPHORESIS:

From mutant strains, that did not degrade BSA at least in one replicate or there were intermediate results in 96-well plate experiment, they were not able to degrade BSA by gel-based method: *ecm33*Δ, *kex2*Δ, *pbs2*Δ, *pex13*Δ, *rsn1*Δ, *orf19.1567*Δ and *ypt72*Δ. Intermediate result at 24 hours of cultivation in YCB-BSA liquid media: SN250, *pep8*Δ, *orf19.6980*Δ, *neo1*Δ and *orf19.3763*Δ. Mutants that were able to degrade BSA after 24 hours: *pkh2*Δ, *chs7*Δ, *gyp1*Δ, *orf19.433*Δ and *rcy1*Δ.

9.4 Western blot

Western blot determining Sap2 (Sap2 antibody was a gift from Dr. M. Monod) was performed in order to find if the problem with BSA degradation was caused due to problems of Sap2 secretion or if it was a problem of syntesis of the protein. For that we compared cytoplasmic extract and supernatant (Figure 26).



Note: PM – protein marker, numbers – molecular mass (kDa).

Figure 25: Western blot of supernatans and cytoplasmic protein extracts of selected mutants (non-degrading or with intermediate phenotypes in previous experiments. A – Supernatant samples (sup) from YCB-BSA cultures. SC5314 – positive control (presence of Sap2), *ecm33*Δ – negative control (absence of Sap2). B – Supernatant (sup) and cytoplasmic extract samples (cyt) from SD cultures. SC5314 – positive control.

Mutant strains that seemed to be non-degrading or with intermediate results from previous experiments were selected. The aim was to confirm the absence of Sap2 enzyme in supernatant from YCB-BSA liquid media after 24 hours (Figure 26A) and to compare the absence of Sap2 in supernatant and cytoplasmic extract of selected mutant strains growing in SD liquid media – protocol for WB: *pbs2*Δ, *neo1*Δ and *orf19.1567*Δ (Figure 26B).

1 As expected, Sap2 is present in supernatant of the wild type strain (SC5314) and in *rcy1*Δ and *gyp1*Δ supernatants. But it was not present in *ecm33*Δ, *pbs2*Δ, *orf19.1567*Δ, *pep8*Δ *and pex13*Δ. A less intense band can be seen in *neo1*Δ.

2 Sap2 is present in the cytoplasmic extracts of three tested mutants (*pbs2*Δ, *neo1*Δ and *orf19.1567*Δ), but not in supernatant samples. There was insufficient amount of proteins in supernatant, because even in SC5314 sample was not possible to detect Sap2 band (positive control for the presence of Sap2).

10 DISCUSSION

C. albicans is a fungus, which is able to secrete lot of types of enzymes. There are three main groups: proteases, lipases and phospholipase B. The family of proteases are called Saps. It is consisted of ten members. Sap2 is responsible for degrading proteins. It is also an important virulence factor. *C. albicans* uses Sap2 enzyme to obtain nutrients and specifically to utilise BSA, when it is the sole source of nitrogen in YCB-BSA medium.

Sap2 is secreted in the classical secretory pathway. It has *N*-terminal signal peptide and it is synthetized as a proenzyme that is transported into Golgi apparatus, where *kex2*Δ proteinase continues with its processing. Activated enzyme is transported through vesicles to plasma membrane followed by release into extracellular space (Naglik *et al.*, 2003).

In recent work a relevant increase of the number of secreted proteins in *C. albicans ecm33*Δ supernatant in comparison to the wild type was described (Gil-bona et al, 2015b). More interestingly, a few proteins have the opposite behaviour. They were not founded in the mutant. Sap2 was among these proteins. This observation connected with the fact that the secretion of other members of the same family (Sap3, 7, 8, 9 and 99) and the phospholipase family, all of them carrying *N*-terminal signal peptide, was not impaired in the mutant strain, prompted to postulate a relation between *ecm33*Δ and Sap2 secretion mechanism in a step of the main secretory route not used for most of the proteins carrying *N*-terminal signal peptide (a branch of the main route).

In this thesis, the aim was firstly to find out if cell-wall mutants have problems to secrete Sap2 (48 kDa) or it is only a problem of *ecm33*Δ mutant strain. The second objective was the identification of more proteins involved specifically in Sap2 secretion. There was a selection of 40 mutant strains that play role at least in one of four processes (transport, protein folding, vesicle-mediated transport and organelle organisation) plus some, which are related to cell-wall structure (glucan metabolism, chitin, cross-linking) and proteins processing. This could help to better understand the mechanism of Sap2 secretion.

We could see the correlation between the optical densities and the results from SDS-PAGE gel in gel-based protocol. If the mutant strain did not degrade BSA as a sole source of nutrient in YCB-BSA liquid media, OD was much considerably lower than for wild-type (SC5314) and the corresponding supernatant analysis processed with gel electrophoresis showed a band, which belonged to BSA (66.5 kDa), that was not in SC5314 culture. This correlation was previously described (Staib et al, 2008). The most significant results were found after 24 hours of cultivation in YCB-BSA liquid media at 30°C using shaker with speed 200 rpm. After 48 hours, some of the mutant strains started to grow, even after they did not show growth at 24 hours. The possible explanation can be that the other Sap proteins were secreted to degrade BSA that is the sole source of nitrogen, instead of Sap2. The other explanation is that they can compensate the defect by different mechanisms.

We tried to use the fact, that OD_{600} and results on gel are correlated, to optimise the method in 96-well plate, which is faster and quantitative. It is possible to use lower quantities of chemicals, media and samples with this method. It allows testing sixteen mutant strains together with the corresponding controls (blanks of media and positive and negative controls of degradation of BSA). After the optimization of the 96-well plate method we found that both methods are comparable, but 96-well plate method shows more advantages. However, most of mutant strains did not grow well after 24 hours, including wild type strain. Thus, the best time for reading the results from 96-well plate was 48 hours. We counted the average value of OD_{600} gathered from the three replicates for each mutant and wild type strains and subtracted the value of optical density of blank (SD or YCB-BSA liquid media) from each average value.

T-test statistical analysis showed, which mutants had significant differences in growth in SD and YCB-BSA liquid media in 96-well plates after 48 hours of incubation. From the selected mutant strains, a batch presented problems to grow in these conditions: *kex2*Δ*, ecm33*Δ*, neo1*Δ*, orf19.1567*Δ*, pbs2*Δ*, pep8*Δ and*, ypt72*Δ mutant strains Otherwise, *ecm33*Δ, *kex2*Δ*, pbs2*Δ*, pex13*Δ*, rsn1*Δ*, orf19.1567*Δ and *ypt72*Δ mutant strains had problem to grow in flasks.

Mutant strains that did not grow in BSA medium in any of the conditions tested were: ***kex2*Δ, *ecm33*Δ**, ***orf19.1567*Δ**, ***pbs2*Δ**, ***ypt72*Δ**. There were some differences between results from gel and 96-well plate for the following strains: *pex13*Δ, *rsn1*Δ that degrade BSA in 96-well plate but not in the gel, and, on the contrary, *neo1*Δ and *pep8*Δ

that had intermediate results in flasks and in 96-well plate were not able to degrade BSA. It could be caused by different methods and conditions used (different time, volume of media, not shaking in 96-well plate, less access to the oxygen in 96-well plate), or to a possible cross-contamination.

We could confirm the results from Ana Gil-Bona work, in which she tested the ability to degrade BSA for SC5314 and *ecm33*Δ *C. albicans* strains. The *ecm33*Δ mutant strain has altered cell wall and is avirulent in murine models of systemic candidiasis as well. In addition, this mutant strain induces protection in mice from lethal infection caused by *C. albicans* SC5314. One of the reasons, why *ecm33*Δ mutant strain is less virulent, can be the disability to secrete Sap2 (Gil-Bona *et al.*, 2015b). We selected mutants connected with cell-wall and secretion of proteins on the basis of this fact. In general, *ecm33Δ* (RML2U) mutant strain is important for cell wall biogenesis and organization (Weig *et al.*, 2004).

*Kex2*Δ has the direct connection with processing of Sap2 in *C. albicans*. It is a subtilisin-like protease (proprotein convertase) that is responsible for processing aspartyl proteinase (Sap2). Previous work described that different *kex2* mutant secreted active Sap2. However, the enzyme was abnormally processed and secreted at reduced levels (Newport and Agabian, 1997). Our results showed in different assays that this *kex2* mutant is unable to grow in media containing BSA, indicating that if there were some secreted Sap2, it was inactive. Kex2 is also required for hyphal growth and virulence in mice. The defect in Kex2 function may lead to the disability of *C. albicans* to colonize and invade tissues (Newport and Agabian, 1997).

Orf19.1567 is a homologue of VAM6, a guanine nucleotide exchange factor for the GTPase Gtr1p in *S. cerevisiae*. It is responsible for regulating the assembly of SNARE (Soluble *N*-ethylmaleimide-sensitive factor attachment protein receptor) complexes (relevant in the secretory pathway) and is involved in vacuolar membrane fusion and vacuolar biogenesis. It plays role in vesicle-mediated transport and piecemeal microautophagy of the nucleus (Wurmser *et al.*, 2000). This is very relevant, because the *C. albicans* homologue may have similar functions that would be impaired in the mutant making it unable to secrete Sap2. This suggests a role of the transport to vacuole in Sap2 secretion of *S. cerevisiae.* Vam6 also interacts with Ypt7 (Nakamura *et al.*, 1997).

Ypt7 is the *S. cerevisiae* homologue of *C. albicans* ypt72 and we found out that it had problems with the secretion of Sap2 as well. It is required for homotypic fusion events in vacuole inheritance and for endosome-endosome fusion (Haas *et al.*, 1995). Ypt72 is important for vacuolar biogenesis, filamentous growth and virulence (Johnston *et al.*, 2009). Thus, a possible explanation of dysfunction in secretion in these mutants could be that the mutation is directly related to the vacuolar biogenesis that gives us a better understanding of the secretion of Sap2. This is in accordance with data of other pre-vacuolar mutants as v*ps1*Δ or *vps34*Δ that also have defects in proteases secretion (Bernardo *et al.*, 2008, Kitanovic *et al.*, 2005).

Pbs2 is a MAP (mitogen-activated protein) kinase-kinase of the HOG (high-osmolarity glycerol) signalling pathway. The secretion is activated under osmotic and oxidative stress in order to ensure stress adaptation (Van Wuytswinkel *et al.*, 2000). It has also a role in organelle organisation and cell wall biogenesis (Arana *et al.*, 2005).

The assumption, that the disability to degrade BSA is related to defect in secretion of Sap2, was not possible to be confirmed with western blot method. In the supernatants from SD liquid media the proteins were not sufficiently concentrated (no band corresponding with Sap2). Thus, it was not possible to compare the presence of Sap2 in supernatant samples from YCB-BSA and SD liquid media or in the supernatant of SD media with the cytoplasmic extract. We wanted to find out if the defect is in the secretion of Sap2 or if Sap2 is not synthetized in these tested mutants. When Sap2 is in the cytoplasm it is possible to compare it to the secretome. But if Sap2 is missing in cytoplasm it is not a problem of secretion but the problem in the synthesis of the proteins.

For further work, it is important to use a higher amount of the culture to concentrate the supernatant for WB to make a comparison of presence of Sap2 in YCB-BSA and SD liquid media. The production of Sap2 can be stimulated as the BSA is the only nutrient in that media. We can only probe the presence of Sap2 inside the cell but not in secretome.

11 CONCLUSION

The aim of this work was to find *C. albicans* mutants without the ability to produce Sap2 enzyme in YCB-BSA liquid media. First of all, it was necessary to optimise the method and establish the easiest, more productive method to test this production of Sap2.

A set of mutants was chosen from the Noble collection that play a role in certain processes like transport, organelle organisation, vesicle-mediated transport and protein folding as well as mutants defective in protein processing, glucan metabolism and chitin.

The results show different ability to secrete Sap2 enzyme in *C. albicans* mutants. But these results were different in various replicates in some cases in 96-well plate experiment and also in the gel.

Mutant strains with significant problems to degrade BSA in experiment with 96-well plate after 48 hours of cultivation in YCB-BSA liquid media (optimised novel method) were *kex2*Δ*, ecm33*Δ*, neo1*Δ*, orf19.1567*Δ*, pbs2*Δ*, pep8*Δ, and *ypt72*Δ. Mutant strains with significant problems to degrade BSA in the experiment with gel after 24 hours of cultivation in YCB-BSA liquid media were *ecm33*Δ*, kex2*Δ*, pbs2*Δ*, rsn1*Δ*, pex13*Δ*, orf19.1567*Δ, and *ypt72*Δ. Strains with intermediate results were *pep8*, *orf19.6980*Δ*, neo1*Δ*, orf19.3763*Δ and even wild type SN250. It means that in both types of experiments were not able to degrade BSA these mutants – *ecm33*Δ*, kex2*Δ*, pbs2*Δ*, ypt72*Δ and *orf19.1567*Δ*. pbs2*Δ*, ypt72*Δ and *orf19.1567*Δ have not been related with Sap2 secretion before.

Western blot method was useful to proof the existence of Sap2 inside the cell in SD liquid media. But the concentration of proteins in supernatant in that media was insufficient. Therefore, it was not possible to compare the secretion of Sap2 in the supernatant from SD and YCB-BSA liquid media. More experiments are needed to check if mutants that do not secrete Sap2 are able to synthetize it.

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