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Functional analysis of the *Candida glabrata* drug:H^+ antiporters Dtr1 and Tpo4: role in stress resistance and virulence

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Resumo

Regista-se, nas últimas décadas, em estreita relação com o aumento do número de indivíduos imunocomprometidos, uma maior incidência de infeções mediadas por espécies de *Candida* [5]. Ainda que a maioria das infeções seja provocada por *Candida albicans*, é também observado que a emergência de infeções originadas por outras espécies do género *Candida* tem vindo a aumentar [5,6,9], sendo *Candida glabrata* uma das mais frequentemente isoladas e a segunda maior responsável pelos casos diagnosticados de candidiases[6,7,8].

A capacidade intrínseca de resistir às pressões exercidas pelas condições de *stress* impostas pelo hospedeiro é um dos factores que tem contribuído, seguramente, para o sucesso de *Candida glabrata* naquelas infeções [14,15]. Embora não seja capaz de formar hifas verdadeiras, como *C. albicans*, *C. glabrata* consegue, através da formação de biofilmes, aderir às células do hospedeiro, bem como a diferentes superfícies artificiais. Esta capacidade de adesão é, em grande parte, mediada pelas adesinas, nomeadamente as pertencentes à família EPA (Epithelial Adhesin Family), cujos membros CgEpa1, CgEpa6 e CgEpa7 foram já caracterizados [11]. A expressão de enzimas extracelulares, como proteases e hemolisinas, é igualmente crucial no processo de infeção, já que essas enzimas auxiliam a penetração nos tecidos, a evasão do sistema imunitário e a obtenção de nutrientes por parte daquela levedura [12,13]. *C. glabrata* mostrou, ainda, ser capaz de tolerar o confronto com células do sistema imunitário, como os macrófagos, usando-os como local imuno-privilegiado para a replicação, e com péptidos antimicrobianos, produzidos pelas células do hospedeiro para eliminar agentes patogénicos invasores [9,48]. Acresce dizer que a mesma levedura evidenciou, também, a capacidade de sofrer alterações cromossómicas, como perdas de cromossomas, rearranjos, translocações e fusões, que contribuem para uma alta diversidade entre a população que, de outra forma, poderia ser afetada pela sua incapacidade de reprodução sexuada [1,15]. Esta plasticidade genómica serve como mecanismo compensatório que lhe permite adaptar-se a um ambiente nefasto e em constante mudança, como o que poderá encontrar no hospedeiro.

Como agravante, esta espécie é conhecida pela resistência adquirida a antifúngicos usados no tratamento das infeções [56,57]. Revela-se particularmente preocupante o facto dessa ocorrência se dar de forma cada vez mais acelerada, uma vez que, por exemplo, a resistência a equinocandinas e ao fluconazole, por parte de isolados de *C. glabrata,* aumentou de zero casos, entre 2001 a 2004, para 9.3% dos casos entre 2006-2010 [57].

Os mecanismos que explicam estas impressionantes capacidades de resistência a drogas e de resiliência dentro do hospedeiro, que têm vindo a ser estudados, podem, entre outros factores, ser mediados pela capacidade de extrusão de compostos tóxicos por bombas de efluxo, incluindo as pertencentes à superfamília MFS (Major Facilitator Superfamily), exaustivamente estudadas em *S. cerevisiae* [61,62,68]. Devido à proximidade entre *S. cerevisiae* e *C. glabrata*, muitos dos referidos transportadores identificados para *S. cerevisiae* têm homólogos em *C. glabrata* [5,61,62,68]. É o caso dos transportadores Dtr1 e Tpo4, nos quais se centra esta tese.

Em *S. cerevisiae*, o transportador Dtr1, formado por 572 aminoácidos, é sintetizado no retículo endoplasmático e levado para a membrana do pró-esporo, a sua última localização [69]. Na ausência deste transportador, a composição da superfície do esporo é alterada e o transporte, quer da molécula bisformil-ditirosina, importante componente da superfície do esporo, quer de ácidos fracos, é fortemente alterado [69]. A tolerância a estes ácidos é muito importante para a proliferação eficaz, dentro do hospedeiro, das espécies de *Candida,* porque, no trato vaginal, existem concentrações significativas de ácido acético e lático. **[111]**. Sublinhe-se que relações sinergéticas entre a resistência a ácidos fracos e a antifúngicos têm vindo a ser descritas, quer para *C. albicans* quer para *C. glabrata*, reforçando, assim, a importância dos ácidos no contexto da virulência e da infeção [112,113].

No caso do transportador Tpo4, identificado em *S. cerevisiae*, observou-se ser determinante na resistência a poliaminas, pois regula a sua presença no citoplasma das células de

levedura [71]. A assimilação de poliaminas tem vindo a ser estudada para outros transportadores da família Tpo, por exemplo o CgTpo3 de *C. glabrata*, envolvido na resistência a azóis e na extrusão de poliaminas para o meio extracelular [63]. A capacidade de inter-relação, entre a homeostase das poliaminas e a resistência a antifúngicos utilizados nas terapias, ganha especial relevo no contexto da infeção no trato urogenital humano, uma vez que aí existem altas concentrações de diferentes poliaminas. Infere-se, portanto, que a resistência a poliaminas pode ser importante para permitir a colonização do epitélio urogenital e melhorar a capacidade de virulência neste local adverso [63].

Trabalhos anteriores, desenvolvidos no grupo BSRG, demonstraram que ambos os transportadores, CgDtr1 e CgTpo4, pertencentes à família de Drug:H^+ Antiporter, estavam relacionados com a letalidade observada no modelo de infeção *Galleria mellonella*.

Os modelos de infeção, dentro dos quais se insere *Galleria mellonella*, têm-se revelado extremamente importantes no estudo dos mecanismos de infeção por parte de leveduras e bactérias. *G. mellonella* torna-se especialmente relevante porque tem um sistema imunitário muito semelhante àquele que é inato nos mamíferos, onde os hemócitos, semelhantes aos neutrófilos e macrófagos humanos, são capazes de fagocitar e matar agentes patogénicos pela produção de espécies reativas de oxigénio e enzimas líticas [56]. A sua fácil manipulação, a inexistência de constrangimentos no controlo do processo de inoculação, bem como os resultados promissores que advêm da utilização deste modelo, elegem-no como primeira opção, dentro do grupo BSRG, para estudar interações entre agentes patogénicos e o hospedeiro.

Neste trabalho, a caracterização funcional do CgDtr1 e do CgTpo4 foi realizada focando-se no seu papel em respostas ao stresse, relevantes no contexto de infeção e de interação levedura-hospedeiro. A deleção do *CgDTR1* revelou limitar a capacidade de proliferação das células de *C. glabrata*, após a injeção, na hemolinfa de larvas de *G. mellonella.* Adicionalmente, resultados para o mutante de deleção *Δcgdtr1* mostraram concentrações ligeiramente menores de células viáveis dentro dos hemócitos, os correspondentes em *G. mellonella* aos macrófagos humanos, quando comparadas com as observadas nas células *wild-type*. Tomando estes resultados em consideração, foi estudado o papel do CgDtr1 na tolerância aos stresses associados à resposta mediada pelos macrófagos. O transportador CgDtr1 mostrou-se determinante na resistência ao stresse oxidativo e aos ácidos fracos, mas não aos antifúngicos. Foi descrito como localizado na membrana plasmática de *C. glabrata*, usando fusão com a GFP, e implicado no decréscimo de acumulação de ácido acético, marcado radioativamente. O transportador *CgTPO4*, não tendo revelado afetar a proliferação de *C. glabrata* na hemolinfa do modelo *G. mellonella,* foi descrito como interveniente na capacidade de resistência à histatina-5, um péptido antimicrobiano humano, semelhante àqueles que fazem parte do sistema imunitário primário das larvas de *G. mellonella*. Tendo em conta a semelhança com o seu homólogo em *S. cerevisiae*, o CgTpo4 foi também descrito como envolvido na resistência a poliaminas, mas não a antifúngicos. Este transportador foi localizado na membrana plasmática em *C. glabrata*, usando fusão com GFP, e associado ao decréscimo de acumulação de espermidina, marcada radioativamente.

Os resultados obtidos no decurso deste trabalho sugerem que o CgDtr1, na virulência de *C. glabrata* em *G.mellonella,* está associado à sobrevivência da levedura dentro dos hemócitos, pelo controlo da concentração intracelular de ácidos fracos, ao passo que o CgTpo4 está relacionado com a capacidade de resistência a péptidos antimicrobianos.

Estes resultados são promissores na medida em que contribuem para o esclarecimento dos mecanismos intervenientes na relação de *C. glabrata* com o hospedeiro, permitindo, assim, redirecionar o estudo dessas relações e, consequentemente, equacionar novas possibilidades terapêuticas, preferencialmente mais focadas nas relações que, comprovadamente, existem entre o hospedeiro e o agente patogénico.

Palavras-chave: *C. glabrata,* virulência, Antiportador Drug:H^+, *Galleria mellonella*, infeção

Abstract

The incidence of *Candida* sp. related infections has been growing significantly over the last couple of decades, mainly due to the increasing numbers of immunocompromised individuals. Within these species, *Candida glabrata* has become the second most frequent cause of candidiasis. Part of the success of this species is due to its intrinsic ability to withstand the pressure exerted by antifungal drugs and several stress conditions imposed by the host.

Earlier work performed at the BSRG has shown that the CgDtr1 and CgTpo4 transporters, from the Drug:H+ Antiporter family, are involved in *C. glabrata* virulence outcome in the *Galleria mellonella* infection model. In this work, the functional characterization of CgDtr1 and CgTpo4 was undertaken, with focus on their role in infection-relevant stress responses and in host-pathogen interaction. The deletion of *CgDTR1* was found to limit the ability of *C. glabrata* cells to proliferate, upon injection, in the haemolymph of the *G. mellonella* larvae. Additionally, the *Δcgdtr1* deletion mutant was found to reach a slightly lower concentration of viable cells inside haemocytes, the *G. mellonella* correspondent to human macrophages, when compared to the wild-type cells. Taking these results into consideration, the role of CgDtr1 in the tolerance to macrophage associate stresses was studied. CgDtr1 was found to be a determinant of resistance to oxidative and weak acid stress, but not to antifungal drugs. This transporter was found to be localized in the *C. glabrata* plasma membrane, using a GFP fusion, and to mediate the decreased accumulation of radiolabelled acetate. *CgTPO4* was not found to affect *C. glabrata* proliferation in *G. mellonella* haemolymph, but was found to confer resistance to histatin-5, a human antimicrobial peptide, similar to those found to be part of the primary immune-system of *G. mellonella* larvae. Similar to its *S. cerevisiae* homolog, CgTpo4 was also found to confer resistance to polyamines, but not to antifungal drugs. This transporter was found to be localized in the *C. glabrata* plasma membrane, using a GFP fusion, and to mediate the decreased accumulation of radiolabelled spermidine.

Altogether, these results suggest that the role of the CgDtr1 in *C. glabrata* virulence against *G. mellonella* is associated to its contribution to survival inside haemocytes, through the control of intracellular concentration of weak acids, while that of CgTpo4 appears to be related to its role in antimicrobial peptide resistance.

Key-words: *C. glabrata,* virulence, Drug:H^+ antiporter, *Galleria mellonella*, infection

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Abbreviations

ABC – ATP Binding Cassette

AMPs – Antimicrobial peptides

ATP – Adenosine triphosphate

C. albicans – *Candida albicans*

C. glabrata – *Candida glabrata*

C. krusei - *Candida krusei*

CFU – colony forming unit

DHA – Drug:H^+ antiporter

EDTA - Ethylenediaminetetraacetic acid

EEA - Early Endosomal Antigen

EPA - Epithelial Adhesin Family

G. mellonella – *Galleria mellonella*

GM-CSF - Granulocyte-Macrophage Colony-Stimulating Factor

GOF – Gain of Fuction

HPLC – High Pressure Liquid Chromatography

hst5 – Histatin-5

IC – Invasive Candidiasis

IL - Interleukin

INFγ - Interferon gamma

LAMP - Lysosomal-associated Membrane Protein

MFS – Major Facilitator Superfamily

MMB – Minimal Medium Broth

MOPS – 3-morpholinopropane- 1-sulfonic acid

NCAC – Non-*Candida albicans Candida*

OD – Optical density

ORF – Open Reading Frame

PAMPs - Pathogen Associated Patterns

PBS – Phosphate Buffered Saline

PCR - Polymerase Chain Reaction

PRRs - Pattern Recognition receptors

ROS – Reactive oxygen species

rpm – Rotations per minute

RPMI - Roswell Park Memorial Institute medium

S. cerevisiae – *Saccharomyces cerevisiae*

SDB – Sabouraud's Dextrose Broth

SEM – Scanning Electron Microscopy

Syk - Spleen tyrosine kinase

TNF-α - Tumor necrosis factor alpha

YPD – Yeast extract Peptone Dextrose medium

1. Thesis Outline

Candida glabrata is one of the most frequently isolated non- *albicans* pathogenic *Candida* species, accounting for nearly 20% of the infections registered in both Europe and North America [6,7,8]. Its success in causing infection is mainly due to high intrinsic stress tolerance that enables this yeast to resist confrontation with the immune system and withstand prolonged harmful conditions such as nutrient starvation and oxidative stress [14,16,9]. Transporters of the Drug:H+ antiporter family, belonging to the Major Facilitator Superfamily (MFS), well characterized in the model yeast *Saccharomyces cerevisiae*, are known to take part in multi-drug resistance as well as in other important physiological stress response mechanisms, including weak acid and polyamine resistance and transport [58,61,62]. At present, not much is known about these transporters and their role in stress responses and host-pathogen interaction in *C. glabrata*. But for some years, the Biological Sciences Research Group has been characterizing the MFS transporters in both *S. cerevisiae* and, more recently, in *Candida glabrata* [60,61,62,63,68,70]. This work, thus, intends to contribute to the functional characterization of two drug:H+ antiporters, CgDtr1 and CgTpo4, particularly focusing their role in the tolerance to different stresses related to host-pathogen interactions.

The CgDtr1 and CgTpo4 transporters were preliminarily studied by Miguel Cacho Teixeira's team (Santos, R. unpublished results), within the BSRG group, reaching the conclusion that they were both involved in early biofilm development and in the virulence outcome when interacting with the *Galleria mellonella* infection model. In this work, we planned to further evaluate the molecular basis of these observations. The knowledge gathered for the CgDtr1 and CgTpo4 homologs in *S. cerevisiae* [70,71] was further used to complete the functional analysis of their *C. glabrata* counterparts.

This thesis is organized into five chapters. The first is an introduction to the *Candida glabrata* epidemiology and pathology, virulence traits, drug-and stress-resistance.

The second part is composed of the materials and method used for the development of this work, followed by the results, that compose chapter 3.

In chapter 4, the integration of the obtained results is performed, within the framework of current knowledge on the subject.

Finally, the fifth chapter is composed by the future perspectives where the unanswered questions raised by the results are highlighted. In this section proposals for future work are brought to light, with the main purposed of further trying to understand the complex mechanisms underlying the action of the studied drug:H+ antiporters that were found to stand in the cross-road of drug resistance and pathogenesis in *C. glabrata*.

2. Introduction

2.1. *Candida glabrata* as an Opportunistic Pathogen

The haploid yeast *Candida glabrata* (H.W.Anderson) S.A.Mey. & Yarrow, previously known as *Torulopsis glabrata* (H.W.Anderson) Lodder & N.F.de Vries[1], is considered to be part of the *Candida* genus, even though it is phylogenetically closer to *Saccharomyces cerevisiae* (E.C. Hans) Meyen (Figure 1) [2]. Although most *S. cerevisiae* genes have orthologs in *C. glabrata* and similar chromosomal structure [3], *C. glabrata* seems to have lost some genes during the evolutionary process, like those involved in galactose, and some related to phospate, nitrogen and sulfur metabolism, most likely due to its adaptation to the mammalian host [4].

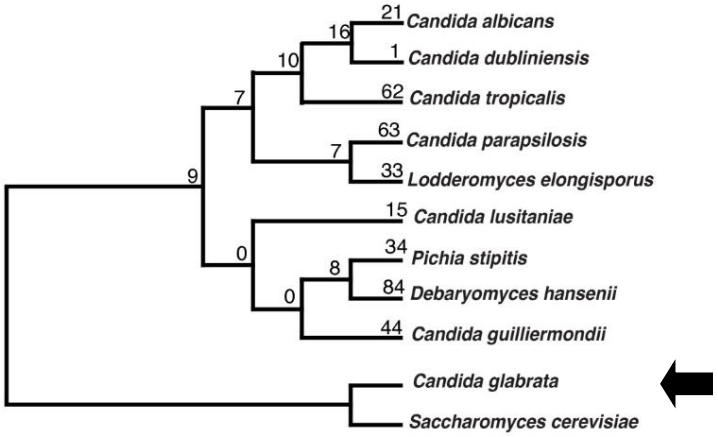

Figure 1: Phylogenetic tree of Candida species representation. (adapted from Fitzpatrick et al, 2010)

The frequency of *Candida* infections has been increasing over the years, largely due to the increasing size of the at-risk populations, which includes transplant recipients, cancer patients and other patients undergoing immuno-suppressive therapy [5]. *Candida* species are among the top 10 most frequently isolated nosocomial bloodstream pathogens, with annual incidence rates of 1.9 up to 4.8 cases, in Europe, and 6.0 to 13.3 cases, in the United States, per 100 000 inhabitants [5].

C. albicans (Robin) Berkhout is the predominant cause of invasive candidiasis, representing 50% to 70% of all cases, however the epidemiology of *Candida* infections has evolved, with longitudinal studies showing that a higher proportion of patients are now infected with non-*albicans* *Candida* species [6]. These include *C. glabrata*, among other examples, which is now accounting for 15 to 20% of the diagnosed cases of candidiasis in Europe [7] and for 20% in North America [8]. This dramatic change has been partly related to the selection of less susceptible *Candida* strains by the widespread use of antifungal drugs [5].

Despite being incapable of switching to true hyphal growth, such as observed in *C. albicans*, *C. glabrata* is a successful pathogen [9] . *C. glabrata* is able to attach to host cells and form biofilms, function that is partly mediated by a large family of epithelial adhesins (Epa proteins), but also by other adhesin-like cell wall-anchored proteins. Adhesins can be divided in sugar-sensitive (lectin-like) or

sugar-insensitive, and they mediate the adherence either to the epithelium or to abiotic surfaces [10]. In *C. glabrata,* only CgEpa1, CgEpa6 and CgEpa7 have been fully characterized [11].

Secretion of extracellular enzymes, such as proteases, lipases, phospholipases, esterases and hemolysins, is considered a virulence factor in *Candida* species. They enable pathogens to penetrate tissues and obtain nutrients [12]. They also play a role in the evasion of the immune system by cleaving immune regulatory proteins or deregulating the cascade-activated homeostatic host systems, such as blood coagulation or antibodies formation pathways [13]. Hemolysins, for instance, induce rupture of erythrocytes, the most common blood cell. Besides the obvious damage this rupture causes, it also releases iron, which is needed for the growth and pathogenicity of yeasts [13].

C. glabrata also appears to undergo chromosomal alterations, including chromosome loss, rearrangements, translocations, chromosome fusions and interchromosomal duplications leading to distinct karyotypes [1]. It is even capable of *de novo* chromosomal generation [15]. All these properties lead to wide population diversity. The genomic plasticity may serve as compensatory mechanism to enable fast adaptation to a changing host environment and compensate for the absence of a sexual cycle [8].

2.2. Immune Evasion Strategies and Macrophage Recognition

C. glabrata has a high intrinsic stress tolerance, which allows her to sustain prolonged starvation periods and to withstand oxidative stress [14]. *In vitro*, this pathogen can tolerate the confrontation with host immune cells, especially macrophages. Indeed, a significant fraction of cells is not only able to survive but also replicate inside human macrophages [9].

Macrophages are professional phagocytes of the monocytic lineage, which can be found in almost all tissues and are abundant in mucosal surfaces. The first contact between a phagocytic cell and a microbe is mediated by host receptors, including pattern recognition receptors (PRRs) which detect conserved basic molecular components of microorganisms, the pathogen associated patterns (PAMPs), and opsonic receptors which recognize opsonized microbes [17]. Recognition of microbial ligands by macrophage receptors activates a series of intracellular signalling pathways that lead to phagocytosis. Engulfed microorganisms are trapped in a plasma membrane-derived vacuole, the phagosome. This premature compartment lacks the ability to break down particles or kill pathogens. During the maturation process, a series of fusion events with compartments of the endosomal pathway takes place, and allows the phagosomes to become more acidic and hydrolytically active. Nutritional limitation and oxidative and non-oxidative antimicrobial mechanisms lead to death or growth restriction of engulfed microorganisms [9].

Since the host defence mechanisms, such as the innate immune phagocytic cells, are multi-facetted, a number of different evasion strategies have evolved. These include avoidance of contact with macrophages, rapid escape from host immune cells, ability to withstand macrophage antimicrobial activities and, most importantly, use of macrophages as an intracellular niche for protection from other immune cells [9]. A frequent strategy is to mask the immune stimulatory

components of the cell wall to avoid recognition and activation by macrophages [18;19]. The cell wall of yeasts is composed, in addition to other elements such as chitin, by β-glucans and proteins protected by an external layer (namely, linear polymers of mannose). β-1,3-glucan has an important role in the recognition by the immune system since it is recognized by the dectin-1 receptor on macrophages [20]. Intact *C. albicans* cells have less interaction with this receptor, since they are protected by the mannan layer. But, when the cell wall suffers any damage, β-1,3-glucan is exposed. When that happens, the dectin-1 receptor quickly recognizes and binds to it, allowing the activation of macrophages and the production of inflammatory cytokines such as TNF-α [21].

Candida glabrata displays a similar response, however, binding to macrophages is also partly mediated by adhesin Epa1 [22]. Therefore, mutants with defects in protein glycosylation are killed more efficiently by macrophages [23,24]. Recently, dectin-2 receptor was regarded as important in host defense against this pathogenic yeast. Knock-out mice for this receptor were more susceptible to infection by *C. glabrata* and phagocytosis decreased in its absence [25]. In addition to recognition of pathogens, another important feature of the immune system is its ability to effectively create an inflammatory response through chemical mediators, especially proinflammatory cytokines such as TNF-α. In *C. glabrata*, TNF-α or other cytokines such as IL1, IL6, IL8 and IFNγ are weakly induced upon infection. The only cytokine that appears to be induced upon infection is GM-CSF [26]. This cytokine functions as a macrophage activator and induces their recruitment. Since *C. glabrata* has the ability to survive and replicate inside macrophages (Figure 2), it is reasonable to believe that attracting more macrophages to the site of infection is an invasion strategy [26].

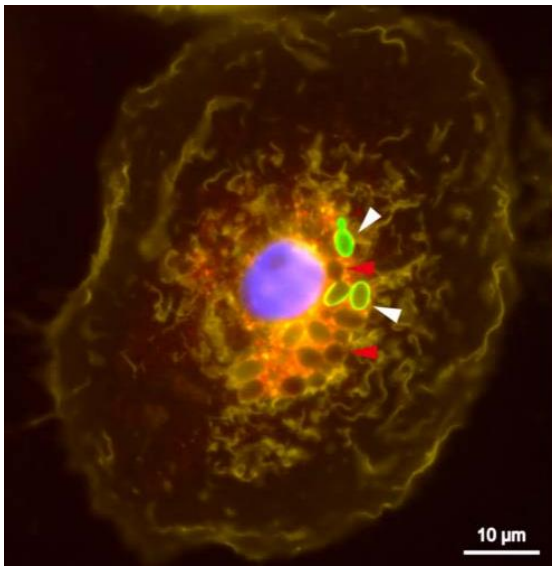

Figure 2: Fluorescent Microscopy Images show *Candida glabrata* replicating inside macrophages

Yeast cells stained with fluorescein isothiocyanate (FITC, shown in green) prior to phagocytosis. The dye is not transferred to daughter cells allowing differentiation between mother cells (white1022 arrows) and newly formed intracellular daughter cells (red arrows) within macrophages after 8 h of co-incubation. Image reported in Kasper *et al*, 2015.

2.3. *Candida glabrata* Survival within the Macrophage

After phagocytosis, microorganisms are internalized in the phagolysosome, the central compartment for the antimicrobial activity of these first line defence cells.

C. glabrata strategy lies in preventing the maturation and acidification of this compartment. So, this yeast resides in a modified phagosomal compartment that acquire early and late endosomal stage markers (EEA 1, early endosomal antigen 1, and LAMP-1, lysosomal-associated membrane protein 1, respectively), but not phagolysosomal markers (for instance, cathepsin D or signal of lysosomal fusion). Therefore, phagosomes that internalize viable yeasts are only weakly acidified [9,27]. If, however, we considered heat-killed yeasts and their exposure to macrophages, they are internalized in a compartment with all phagolysosomal properties. This is indicative that for the modification from phagosome to phagolysosome either a heat-labile surface factor is required or the metabolic activity of the yeast is needed [9,27].

In addition, it was recently observed for *C. albicans*, that the metabolization of amino acids as an alternative carbon source, with consequent ammonia extrusion, is implicated in the alkalinization and neutralization of the phagosome environment [9]. *Candida glabrata* is also able to alkalinize its environment *in vitro* when resorting to the use of amino acids in the absence of glucose. The Mnn10 and Mnn11 mannosyltransferases are important for this environment alkalinization and prevention of phagosome acidification, suggesting that they are included in the strategy to raise the environmental pH [9].

Another possible strategy lies within the dectin-1 mediated Syk signaling. *C. glabrata* viable cells can induce a less pronounced Syk activation, implying that a reduced signalling could underlie the delivery of viable yeasts to non-matured phagosomes [9]. Still, it is not entirely clear or accepted that this yeast needs to prevent phagosome maturation or modify its pH to survive. The truth is that, *in vitro,* this yeast can grow unaffected when exposed to acidic pH, as low as 2.0 [28]. Even so, the combination of pH with an arsenal of antimicrobial properties within the macrophage may restrict their survival. That is, it is more likely that the observed effects result from a combination of factors.

The production of reactive oxygen species (ROS), along with a low pH value, represent a central aspect of the antimicrobial response in macrophages. Its production is driven by the NADPH oxidase complex that generates superoxide, hydroxyl anions and radicals [29]. However, *C. glabrata* has a high resistance to oxidative stress. This resistance is partly mediated by Cta1 catalase expression [30]. Nevertheless, *in vitro* response to oxidative stress also includes thioredoxin peroxidases (Tsa1 and Tsa2) thioredoxin reductases (Trr1 and Trr2), thioredoxin cofactor (Trx2), glutathione peroxidase (Gpx2) and superoxide dismutases (Sod1 and Sod2) [31]. During *in vitro* experiences of *C. glabrata* interaction with macrophages, Sod1 was found to confer resistance to killing in the absence of Yap1 mediated signalling. In addition, the *CTA1*-reporter gene was found to be induced after phagocytosis, indicating that macrophage interaction leads to oxidative stress sensing by *C. glabrata* and to the subsequent activation of Cta1 [9]. Studies on human and murine macrophages have shown that *C. glabrata* suppresses ROS production by phagocytes. Even so, NADPH oxidase is activated by recognition and phagocytosis of microorganisms, even before the

phagosome is sealed [9]. Thus, it is not expected that incomplete phagosome maturation cause a decrease in ROS induction. ROS suppression is currently viewed as an active down-regulation of macrophage ROS production by this pathogenic yeast, as an immune evasion strategy [27,9]. While a correlation between phagocytosis-associated oxidative metabolism and killing has been described for *C. albicans*, the role of ROS in *C. glabrata* killing is still unclear. The high intrinsic resistance to oxidative stress along with the fact that inhibition of ROS production in macrophages did not increase yeast survival [32], suggests that ROS may play a secondary role in direct killing of *C. glabrata*.

To be able to survive and replicate in the phagosome, *C. glabrata* has to adapt to the surrounding environment , not only low pH values and oxidative stress but also nutritional restrictions. This includes adjustment to the use of alternative carbon sources, as well as nitrogen deprivation [9]. The transcriptional response of *C. glabrata* to phagocytosis reflects up-regulation of genes encoding for enzymes of the glyoxylate cycle, gluconeogenesis, and β-oxidation of fatty acids, as well as down-regulation of glycolysis. These responses suggest the triggering of alternative carbon source metabolism [33,34]. Also, down-regulation of protein synthesis and up-regulation of amino acid bio-synthetic pathways, and amino acid and ammonium transport genes, suggests that the yeast undergoes deprivation of nitrogen. In addition, methylcitrate cycle genes, important for the degradation of fatty acid chains which enables the use of lipids as alternative carbon source, was found to be up-regulated in these conditions [33,34].

Aside from carbon and nitrogen, trace elements, such as iron, are important for *C. glabrata* growth. Iron is an essential transition metal which serves as a cofactor for many enzymes and is required for numerous biochemical processes, including cell respiration and metabolism, oxygen transport and DNA synthesis [35]. Thus, it is not surprising that the host sequesters iron from extracellular spaces, to challenge organisms with micronutrient limitations, a process known as nutritional immunity [36]. *C. glabrata* does not use ferritin or transferrin as iron sources, and is not able to use heme or hemoglobin. However, it expresses one siderophore-iron transporter called Sit1. Sit1 mediated binding increases fitness and survival during exposure to macrophages. Iron availability and acquisition are important determinants of intracellular survival and replication in macrophages. Recent studies showed that 11 out of 23 mutants identified for reduced survival within macrophages showed defects in growth under iron limitation [32].

Depending on the organism there are a number of possible strategies to evade the host, and that also includes possible exit strategies from macrophages. These strategies include non-lytic exocytosis, observed for *C. albicans* and *C. neoformans* (San Felice) Vuillemin (assexual morph of *Filobasidiella neoformans* Kwon-Chung), induction of host cell death by apoptosis or pyroptosis or caspase-dependent early lytic pro-inflammatory cell death [9]. After phagocytosis, *Candida albicans* rapid hypha formation is activated. Its triggering is important for initiation of inflammatory responses and also mechanical damage and escape from the macrophage [9]. *C. glabrata* grows predominantly in the yeast form and only forms pseudohypha under certain conditions, particularly nitrogen deprivation. However, microevolution experiments with permanent exposure of *C. glabrata* to macrophages for several months selected populations with genetic changes that caused a higher pseudohypha-like growth, greater mechanical damage of macrophages and faster escape [37].

During the interaction with human macrophages derived from monocytes, *C. glabrata* remains intracellular for 2-3 days without causing obvious damage to macrophages or inducing cell death [9].The replicating yeast are still surrounded by membrane and are not released in to the cytoplasm, as indicated by detection of LAMP-1 (lysosomal-associated membrane protein) around the ingested yeast cells after 24 hours. After 2-3 days, macrophages with increased fungal burden were observed to lyse and release yeasts into the surrounding medium [27]. Even so, the time of macrophage burst is dependent on the initial yeast-to-macrophage ratio and the macrophage cell type [9].

2.4. Virulence traits for the establishment of infection: biofilm formation and antimicrobial peptide resistance

Besides phagocytosis, in the event of *Candida* infection, the adherence to host surfaces is required for colonization and infection establishment [38].Adherence contributes to the organism persistence inside the host, which is crucial for the disease development strategy. *Candida* species are well known for its adherence capacities and biofilm formation, not only within the host, but also attached to medical devices [38,39].

Cell surface proteins, like adhesins encoded by the EPA gene family, are known to promote specific adherence. But the initial attachment of *Candida* cells to the host is not only promoted by cell division, proliferation and adherence, but is also dependent on biofilm development [40]. Biofilms are surface-associated communities of microorganisms involved by an extracellular matrix, and they present the most prevalent growth form amoung different microorganisms [38,41]. Since this structure has been known to confer resistance to antifungal drugs, by limiting drug penetration, and protection from the host immune system, biofilm formation is considered one of the most important virulence factors used by pathogenic yeast cells, such as *C. glabrata* [42,43]. Furthermore, they have also been associated with higher morbidity and mortality rates [44].

C. glabrata is known to produce lower quantities of biofilm mass when compared with other NCAC (non-*C. albicans Candida*) species but, is has been showed that higher biofilm biomass is produced, by this yeast, on artificial surfaces, such as silicon in the presence of urine, then in SDB medium when compared with *C. parapsilosis* (Ashford) Langeron & Talice, 1932 or *C. tropicalis* Berkhout, 1923 [41,45]. *C. glabrata* biofilm matrices were already characterized has having high levels of both proteins and carbohydrates, concordant with what has been seen for *C. albicans* whose biofilm matrix which is composed mainly by carbohydrates, proteins, phosphorus and hexosamines [41]. It is also important to highlight that *C. albicans* biofilm is formed by a mixed population of budding-yeast and hyphae, but no filamentation has yet been observed in *C. glabrata* biofilms [46,47].

In order to fully establish a functional biofilm, the first step is the adherence of yeast cells to host surfaces or abiotic medical devices. After adherence, formation of colonies, secretion of extracellular polymeric substances, maturation in a three-dimensional structure and cell dispersion have to occur. In the following scheme (Figure 3) the various steps behind biofilm formation are detailed.

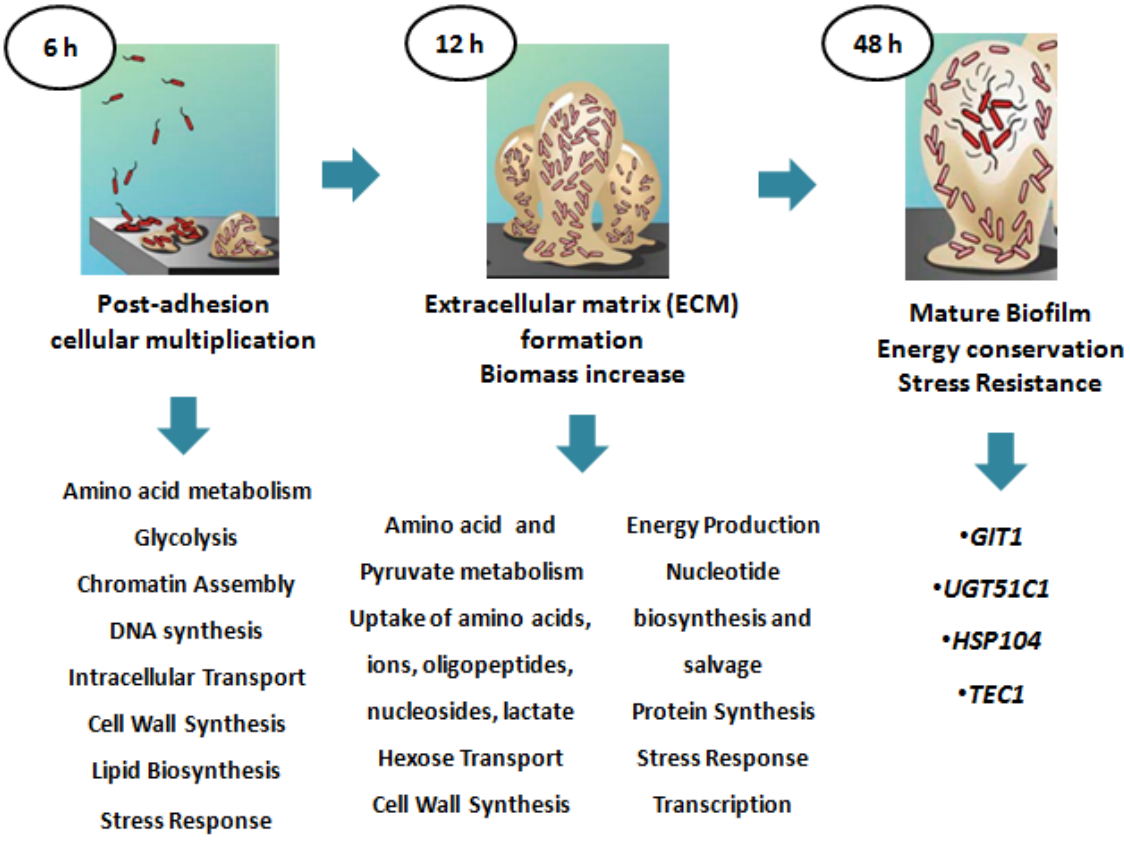

Figure 3: Scheme representing the three major phases for biofilm development. Resumed phases and main factors involved in biofilm formation are presented. Phases from adhesion to mature biofilm development are specified taking into account participating molecules and main mechanisms involved. (adapted from Yeater *et al,* 2007)

Besides biofilm development, another important virulence trait is the ability to resist the action of antimicrobial peptides. Antimicrobial peptides are small, positively charged, amphipathic molecules that may be composed by a variety of amino acids and have various lengths (from 6 to 100 amino acids) [48]. In humans, the most prominent innate antimicrobial peptides are the cathelicidins and defensins, produced by immune system cells, and the histatins produced and secreted into the saliva by the parotid, mandibular and submandibular salivary glands [48].

Most antimicrobial peptides work directly against microbes through a mechanism that involves membrane disruption and pore formation, that eventually leads to the efflux of essential ions and nutrients [49]. The mechanism behind the association with and permeabilizing off the microbial cell membranes is not entirely clear, but these peptides are proposed to bind to the cytoplasic membrane, creating micelle-like aggregates, leading to a disruptive effect (Figure 4) [48]. Nonetheless, studies have been indicating the presence of additional or complementary mechanisms such as intracellular targeting of cytoplasmic components crucial to cell physiology functions [48]. Thus, the initial interaction between peptides and microbial cell membranes would allow them to penetrate inside the cells to bind to intracellular molecules, resulting in the inhibition of cell wall biosynthesis, and processes involving DNA, RNA and protein synthesis and function [50,51] . These modes of action, combined with a broad

range of lethal activity in a short period of time, corroborate the hypothesis that antimicrobial peptides are excellent candidates for the development of novel therapeutic agents [48]. Therefore, the acquisition of resistance against their killing activity is crucial for yeasts, and other pathogenic microorganisms, to establish a successful infection.

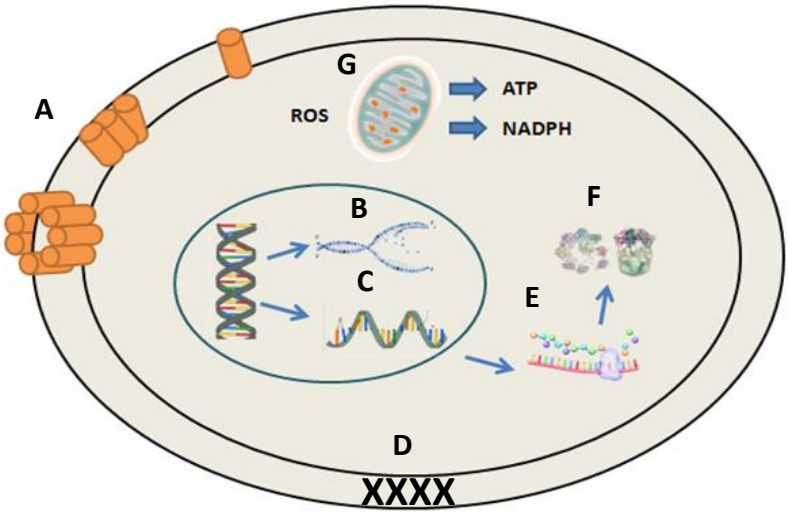

Figure 4: Mechanisms modes of action for antimicrobial peptides in microbial cells. (A) Disruption of cell membrane integrity: starting with random insertion into the membrane, followed by alignment of hydrophobic sequences and removal of membrane sections and pore formation. (B) Inhibition of DNA synthesis. (C) Blocking of RNA synthesis. (D) Inhibition of enzymes necessary for linking of cell wall structural proteins. (E) Inhibition of ribosomal function and protein synthesis. (F) Blocking of chaperone proteins necessary for proper protein folding. (G) Targeting of mitochondria: starting with inhibition of cellular respiration and induction of ROS formation and disruption of mitochondrial cell membrane integrity and efflux of ATP and NADH. (adapted from Peters *et al*, 2010).

2.5. Infection Models to Study Virulence of *Candida glabrata*

Historically, there has been little interest in developing animal models for *C. glabrata* infections, despite the emergence of both systemic and mucosal infections [52]. Even so, different mouse models of oral, vaginal, gastrointestinal, intraperitoneal and intravenous bloodstream infections have been developed [53]. During systemic mouse infection, fungal dissemination spreads to different organs, such as brains, kidneys, lungs and even heart, which shows that *Candida glabrata* is highly adaptable and able to colonize many niches [52,53]. Grating this, different mouse models have been applied to study the virulence of *C. glabrata* gene deletion mutants. These studies analyze fungal burden and not host mortality, due to low mortality of mice after infection, since even high fungal burden still causes low tissue damage and low inflammation responses (Kasper *et al;* 2015). That being established, the respective genes should be termed fitness factors rather than virulence factors of *C. glabrata* [9].

Additionally, non-vertebrate models, such as *Drosophila melanogaster, Caenorhabditis elegans* and *Galleria mellonella* have also been established for this yeast. These offer a number of advantages over mammalian vertebrate models due to economy of size, ease of handling and ethical issues [54,55].

Galleria mellonella larvae, for instance, bears several advantages: they are cheap to acquire and easy to keep, their size simplifies infection and the inoculation process via syringe allows control

of the amount of pathogen applied. Plus, the larvae can be incubated at a range of various temperatures which may go up to 37ºC [55].

The larval immune system has high similarity to the mammalian innate immune system. The haemocytes, phagocytic cellular components similar to neutrophils, are able to phagocyte and kill pathogens by producing reactive oxygen species and lytic enzymes [56]. Furthermore, the humoral response to infecting microbes involves antimicrobial peptides which were shown to be induced by fungal pathogens, and differ from those induced by bacterial pathogens [55]. This feature is very important because it allows comparison of data obtained in *Galleria* virulence studies and murine models. Moreover, experiments with *G. mellonella* grant fast data acquisition, usually with results within one or two weeks [55]. Notwithstanding, there are also disadvantages of this model. After all, the genome of *G. mellonella* has not been fully sequenced and there are no mutant strains available to functionally study host response. Plus, they do not have adaptive immune systems, which disable further immune response studies. Even so, *Galleria mellonella* is a useful tool and potent alternative infection model to study fungal pathogens.

2.6. Antifungal Drug Resistance

During the past three decades antifungal drug resistance became more common and a serious concern to the medical community [1]. The proportion of azole resistance in clinical isolates across several countries has been shown to increase in the period from 2001 to 2007 [56]. Furthermore, resistance to echinocandins of fluconazole-resistance *C. glabrata* isolates was shown to have increase from no cases between 2001-2004 to a 9,3% frequency in the 2006-2010 time period [57]. This supports the idea that drug resistance in *C. glabrata* is developing fast.

Classes of antifungals are categorized based on their mechanisms of action. Azoles inhibit the cytochrome P450-dependent enzyme lanosterol-demethylase which is crucial for the biosynthesis of ergosterol. If ergosterol synthesis is affected, the resulting cell membrane dysfunction impairs signalling and transport processes [1]. Polyene antifungals, such as amphotericin B, interact with ergosterol in the cell membrane creating pores within the cell membrane causing small molecules to leak, resulting in cell death [47]. 5-Flucytosine is an antifungal that interferes with protein synthesis. Upon entering the cell through a cytosine permease, this drug is converted into a nucleotide analogue, 5-flurouracil, which is incorporated into RNA, subsequently interfering with the synthesis of proteins [38]. Finally, the more recently introduced antifungals, echinocandins, have become the first line therapy for invasive candidiasis. They are able to inhibit β-(1,3)-D-glucan synthase activity, preventing the synthesis of β-(1,3)-D-glucan, essential for the composition of the fungal cell wall [47].

Understanding the mechanisms of antifungal resistance is the key to unravel new therapeutic options. Resistance may be classified as: primary resistance, when strains are unaffected by the antifungal without prior exposure; or secondary resistance, when strains acquire resistance after exposure to the drug. Secondary resistance is, by far, the most commonly occurring phenomenon among *Candida* species [1].

C. glabrata, when compared to other *Candida* species, is the least susceptible to azoles. The mechanisms behind azole resistance in *C. glabrata* include changes in P-450 lanosterol demethylase enzyme, encoded by *ERG11*, which results in loss of the drug affinity and/or overexpression of *ERG11*, or activation of drug extrusion catalysed by membrane transport proteins of the ATP binding cassette (ABC) superfamily or the Major Facilitator Superfamily (MFS) [1]. Azoles enter the cell by facilitated diffusion and bind to the iron present in the heme group of the Erg11 enzyme [58,59]. This N-Fe connection restrains the activation of oxygen, which is necessary for the demethylation of lanosterol, causing a blockage in the ergosterol production. Consequently, a toxic sterol, known as 14-α-methyl-3,6-diol, accumulates, causing severe stress in the plasma membrane. This results in a fungistatic behaviour in the yeast cells [58].

CDR1 and *CDR2* genes, encoding drug efllux pumps of the ABC superfamily, are up-regulated in response to azole exposure. The transcription factor, Pdr1, that regulates the expression these genes, is activated through direct binding to the azole drug molecule, promoting the expression of the drug efflux pump encoding genes, being a key azole resistance determinant. Disruption and/or removal of the *PDR1* gene was found to increase susceptibility to azoles [56]. It was also found that Gain Of Function (GOF) mutations in *PDR1*, identified in azole resistant clinical isolates, enhance azole resistance and even virulence in mice models. More recently, a few multidrug transporters of the Major Facilitator Superfamily, suggested to act as Drug:H+ Antiporters (DHA), have also been shown to confer resistance to azole drugs, especially imidazoles [60,61,62,63].

Little is known about the exact mechanism for resistance to polyene antifungal drugs in *C. glabrata.* A clinical isolate susceptible to polyene treatment was observed to have severe changes in the sterol composition of its membrane [64]. This isolate had accumulated numerous sterol intermediates that were still able to maintain membrane viability. Further studies showed also that nonsense mutations, as opposed to missense, in *ERG6* gene generated altered sterol composition in the cell membrane [65].

Unlike azole antifungals, secondary resistance to echinocandins has conclusively been unrelated to the drug-efflux mechanism as they prove to be poor substrates for most multidrug efflux transporters [66]. Instead, echinocandin resistance is caused by interference between the drug and the β-1,3-D glucan synthase. The target subunits for this enzyme are encoded by three genes: *FSK1*, *FSK2* and *FSK3* [67]. Point mutation in the *FSK1* and *FSK2* are documented as being responsible for echinocandin resistance in *Candida glabrata.* Mutations in "hot spots" of these genes cause a high prevalence of amino acid substitutions conferring echinocandin resistance [64].

2.7. The putative *Candida glabrata* Multidrug Resistance Transporters CgDtr1 and CgTpo4

In this study, the uncharacterized drug:H+ antiporters Dtr1 and Tpo4, encoded by ORF *CAGL0M06281g* and *CAGL0L10912g*, respectively, were studied in the context of their role in *C. glabrata* virulence.

First studied in *S. cerevisiae,* MFS transporters related to mutidrug resistance were grouped into two families: Drug:H+ antiporter family (DHA1), including 12 transporters, and Drug:H+ antiporter family 2 (DHA2), including 10 transporters. The difference between the two groups is established based on the number of transmembrane domains exhibited by each family, 12 for the DHA1 family members and 14 for the DHA2 [68]. In *Candida glabrata* we find 10 predicted members of the DHA1 and 5 of the DHA2 families. From these, a total of 6 seem to play a role in drug resistance and detoxification of the cell, but none of the DHA2 is, up to date, described as being involved in this phenomenon [61,62].

Studies in the *S. cerevisiae* homologs of Dtr1 and Tpo4 were used to guide the functional analysis of their *C. glabrata* counterparts. *S. cerevisiae* Dtr1 is synthesized in the endoplasmic reticulum and then transits to the Golgi complex before reaching the prospore membrane, it's final location. Dtr1 is formed by 572 amino acids with 12 predicted transmembrane domains and a large cytoplasmic loop in the middle [69]. It is encoded by the open reading frame YBR180W, and is described as being a membrane dityrosine transporter [70]. This role is related to the translocation of bisformil-dityrosine from the cytoplasm of the prospore to the maturing spore wall during the wall formation process. According to Ferder and colleagues, in the absence of *DTR1*, the amount of D-L dityrosine (present in the spore surface) is reduced to 65% and the racio between this surface macromolecule and the soluble spore precursors (DL/LL racio) changes from 0,6 to 0,2. The change of this value does not seem to have a major consequence in the sporulation process. Even so, it was observed, by the same authors, that the Dtr1 transporter is essential for bisformyl dityrosine molecule being able to pass through the plasmatic membrane, on its way to the prospore membrane [70]. Besides dityrosine, Dtr1 was also shown to play a role in the export of weak acid conjugated bases, its expression conferring resistance to this class of compounds [70].

In the case of Tpo4, it was identified in S. cerevisiae as a determinant of resistance to polyamines [71]. The polyamine content present in the cytoplasm of yeast cells appears to be tightly regulated by Tpo4, but also paralogous transporters including Tpo1, Tpo2 and Tpo3, that guarantee an optimal intracellular polyamine content [71].

3. Materials and Methods

3.1. Strains and Growth Media

Considering the thesis objectives, several strains of *Candida glabrata* where used and are described in Table 1.

Table 1: *C. glabrata* strains used to assess stress resistant responses and host-pathogen interactions

Strain	Genotypic Background
Wild-type	Kchr606
Δcgdtr1	Kchr606_Δdtr1
Δcgtpo4	Kchr606_Δtpo4
Wild-type	L5U1
Wild-type	By4741
Wt + CgTpo4	By4741_tpo4
Wt + CgDtr1	By4741_dtr1

Cells of *C. glabrata* were cultured in rich Yeast extract Peptone Dextrose (YPD) medium, containing *per* litre: 20g D(+)-glucose (Merck), 20g of bacterial-peptone (LioChem, Inc.) and 10g of yeast extract (Difco) and also in Minimal growth medium (MMB) medium, containing *per* litre: 20g D(+)-glucose (Merck), 2,7g ammonium sulphate (Difco) and 1,7g of Yeast-Nitrogen-Base without amino acids (Difco). When needed, solid media was obtained by supplementation with 20g/L of agar (NzyTech). In what concerns the L5U1 strains, growth media was supplemented with leucine 20x concentrated and 1mM of copper solution. As for BY4741, solid and liquid media, was supplemented with an amino acid solution containing, per liter, 20 mg methionine, 20 mg histidine, and 60 mg leucine. Furthermore, when using this strain in order to express differences between mutated strains, the sugar composition of MMB changed from 20g/L D(+)-glucose (Merk) to 1g/L D(+)-glucose (Merk) plus 10g/L D(+)-galactose (Sigma).

Roswell Park Memorial Institute medium (RPMI) 1640 and Sabouraud Dextrose Broth (SDB) media were used for biofilm assays. Such media preparation took 10,4 g of RPMI 1640 (Sigma-Aldrich), 34,5g of MOPS (Sigma) and 18g of D(+)-glucose (Merk) dissolved in 1L of deionised water, adjusted to pH 4.0 and 40g of D(+)-glucose (Merk) and 10g of peptone (LioChem) *per* litre of deionised water, adjusted to pH 5.6, respectively.

3.2. Cloninf of the *C. glabrata CgDTR1 and CgTPO4* genes

3.2.1. Cloning of the *C. glabrata CgDTR1* gene (ORF *CAGL0M06281g*)

The pGREG576 plasmid from the Drag & Drop collection [73] was used to clone and express the *C. glabrata* ORF *CAGL0M06281g* in *S. cerevisiae*, as described before for other heterologous genes [74]. pGREG576 was acquired from Euroscarf and contains a galactose inducible promoter (GAL1), the

yeast selectable marker URA3 and the GFP gene, encoding a Green Fluorescent Protein (GFPS65T), which allows monitoring of the expression and subcellular localization of the cloned fusion protein. CAGL0M06281g DNA was generated by PCR, using genomic DNA extracted from the sequenced CBS138 *C. glabrata* strain, and the following specific primers: 3' – GAATTCGATATCAAGCTTATCGATACCGTCGACA*ATGAGCACCTCCAGCAACAC* - 5' and 3' – GCGTGACATAACTAATTACATGACTCGAGGTCGAC*TCAGAAACTGTCTTTAACCC* - 5'. The designed primers contain, besides a region with homology to the first and last 22 nucleotides of the *CAGL0M06281g* coding region (italic), nucleotide sequences with homology to the cloning site flanking regions of the pGREG576 vector (underlined). The amplified fragment was co-transformed into the parental *S. cerevisiae* strain BY4741 with the pGREG576 vector, previously cut with the restriction enzyme SalI, to obtain the pGREG576_*CgDTR1* plasmid. Since the GAL1 promoter only allows a slight expression of downstream genes in *C. glabrata*, to visualize by fluorescence microscopy the sub-cellular localization of the *CgDTR1* gene in *C. glabrata*, a new construct was obtained. The GAL1 promoter present in the pGREG576_*CgDTR1* plasmid was replaced by the copper-induced MTI *C. glabrata* promoter, giving rise to the pGREG576_MTI_*CgDTR1* plasmid. The MTI promoter DNA was generated by PCR, using genomic DNA extracted from the sequenced CBS138 *C. glabrata* strain, and the following specific primers: 3' - TTAACCCTCACTAAAGGGAACAAAAGCTGGAGCTC*TGTACGACACGCATCATGTGGCAATC* - 5' and 3' - GAAAAGTTCTTCTCCTTTACTCATACTAGTGCGGC*TGTGTTTGTTTTTGTATGTGTTTGTTG* - 5'. The designed primers contain, besides a region with homology to the first and last 19 nucleotides of the first 1000 bp of the MTI promoter region (italic), nucleotide sequences with homology to the cloning site flanking regions of the pGREG576 vector (underlined). The amplified fragment was co-transformed into the parental strain BY4741 with the pGREG576_*CgDTR1* plasmid, previously cut with SacI and NotI restriction enzymes to remove the GAL1 promoter, to generate the pGREG576_MTI_*CgDTR1* plasmid. The recombinant plasmids pGREG576_*CgDTR1* and pGREG576_MTI_*CgDTR1* were obtained through homologous recombination in *S. cerevisiae* and verified by DNA sequencing.

3.2.2. Cloning of the *C. glabrata CgTpo4* gene (ORF *CAGL0L10912g*)

The pGREG576 plasmid from the Drag & Drop collection [73] was used to clone and express the *C. glabrata* ORF *CAGL0L10912g* in *S. cerevisiae*, as described before for other heterologous genes [74]. pGREG576 was acquired from Euroscarf and contains a galactose inducible promoter (GAL1), the yeast selectable marker URA3 and the GFP gene, encoding a Green Fluorescent Protein (GFPS65T), which allows monitoring of the expression and subcellular localization of the cloned fusion protein. CAGL0L10912g DNA was generated by PCR, using genomic DNA extracted from the sequenced CBS138 *C. glabrata* strain, and the following specific primers: 3' – GAATTCGATATCAAGCTTATCGATACCGTCGACA*ATGGCCGGTACAAATCAAG*- 5' and 3' – GCGTGACATAACTAATTACATGACTCGAGGTCGAC*CTATACCATTCTAGAGGAG* - 5'. The designed primers contain, besides a region with homology to the first and last 22 nucleotides of the *CAGL0L10912g* coding region, nucleotide sequences with homology to the cloning site flanking regions of the pGREG576 vector (underlined). The amplified fragment was co-transformed into the parental *S. cerevisiae* strain BY4741 with the pGREG576 vector, previously cut with the restriction

enzyme SalI, to obtain the pGREG576_*CgTPO4* plasmid. Since the GAL1 promoter only allows a slight expression of downstream genes in *C. glabrata*, to visualize by fluorescence microscopy the sub-cellular localization of the *CgTPO4* gene in *C. glabrata*, a new construct was obtained. The GAL1 promoter present in the pGREG576_*CgTPO4* plasmid was replaced by the copper-induced MTI *C. glabrata* promoter, giving rise to the pGREG576_MTI_*Cgtpo4* plasmid. The MTI promoter DNA was generated by PCR, using genomic DNA extracted from the sequenced CBS138 *C. glabrata* strain, and the following specific primers: 3' - TTAACCCTCACTAAAGGGAACAAAAGCTGGAGCTC*TGTACGACACGCATCATGTGGCAATC* - 5' and 3' - GAAAAGTTCTTCTCCTTTACTCATACTAGTGCGGC*TGTGTTTGTTTTTGTATGTGTTTGTTG* - 5'. The designed primers contain, besides a region with homology to the first and last 19 nucleotides of the first 1000 bp of the MTI promoter region (italic), nucleotide sequences with homology to the cloning site flanking regions of the pGREG576 vector (underlined). The amplified fragment was co-transformed into the parental strain BY4741 with the pGREG576_*CgTpo4* plasmid, previously cut with SacI and NotI restriction enzymes to remove the GAL1 promoter, to generate the pGREG576_MTI_*CgTpo4* plasmid. The recombinant plasmids pGREG576_*CgDTR1* and pGREG576_MTI_*CgTpo4* were obtained through homologous recombination in *S. cerevisiae* and verified by DNA sequencing.

3.3. Virulence assessment using the *Galleria mellonella* infection model

3.3.1. Survival and Proliferation Assessment Assays

Galleria mellonella larvae were reared on a pollen grain diet at 25ºC in darkness. Larvae weighting 250 ± 25 mg were used in survival assays, where the larvae infection was performed as described previously [116]. *C. glabrata* strains were cultured in YPD up to stationary phase and harvested by centrifugation in order to achieve an yeast suspension. Each caterpillar was injected with 3.5 µL of yeast suspension (with approximately $5x10^7$ cells *per* injection) via the last left proleg. Following injection, 10 and 30 larvae, for survival and proliferation assays, respectively, were placed in Petri dishes and maintained in the dark at 37ºC over a period of 72 hours. Control larvae were injected with PBS (pH 7.4). Regarding the survival assays, caterpillars were considered dead when they displayed no movement in response to touch. For proliferation assays, hemolymph was recovered with a microsyringe after 1, 24 and 48 hours and plated in YPD medium for CFU counting. Larvae injections and hemolymph recoveries were performed in collaboration with Dra. Dalila Mil-Homens, Biological Sciences Research Group of the Institute for Bioengineering and Biosciences.

3.3.2. Haemocyte Interaction Assays

3.3.2.1. *In vitro* cultivation of haemocytes of *G. mellonella.*

To isolate *G. mellonella* haemocytes, hemolymph was collected from larvae previously anesthetized on ice and surface sterilized with ethanol by puncturing the larval abdomen with a sterile needle.The outflowing hemolymph was immediately transferred into a sterile microtube containing anticoagulant buffer (98 mM NaOH, 145 mM NaCl, 17 mM EDTA, 41 mM citric acid [pH 4.5]) in a 1:1 proportion. The hemolymph was centrifuged at 250 × g for 10 min at 4°C to pellet haemocytes. The supernatant was

taken off, and the pellet was washed twice with 0.9% NaCl and centrifuged at 250 × g for 5 min at 4°C. The hemocyte pellet was then suspended gently in 1 ml of Grace insect medium (GIM) (Sigma) supplemented with 10% fetal bovine serum, 1% glutamine, and 1% antibiotic or antimycotic solution (10,000 U penicillin G, 10 mg streptomycin, 25 mg/liter amphotericin B). Suspended haemocytes were counted with a hemocytometer and incubated at 26°C in 24-well plates at a concentration of 2 × 105 cells/ml. Monolayers of primary *Galleria* hemocytes were used for experiments the next day. All preparations and assays were carried out under sterile conditions.

3.3.2.2. Determination of *in vitro* yeast load of haemocytes.

Cultures of *C. glabrata* cells were grown in YPD medium until mid-exponential phase (OD_{600nm} = 0.4-0.6). The optical density of the cultures was measured, and the appropriate volume was collected to have 7 × 10^2 cells/ml in 0.9% NaCl. *Galleria* haemocyte monolayer medium was replaced with GIM without antimucotics, and then cells were infected with the yeast suspensions. After 1 h of infection at 37°C, the haemocytes were carefully washed twice with cell culture medium, followed by the addition of GIM. The quantification of viable intracellular yeast cells was achieved 1, 4, 24 and 48 hours after infection. Cell monolayers were lysed with 0.5% Triton X-100, and CFU were determined by plating dilutions of cell lysates on YPD-agar plates followed by incubation at 30°C for 24 h.

3.4 - Candidacidal assay of Histatin-5

To evaluate the effect of *CgDTR1* and *CgTPO4* expression in Histatin-5 resistance, wild-type, ∆cgdtr1 and ∆cgtpo4 *C. glabrata* mutants were batch cultured in YPD until mid-exponential phase (OD_{600nm} = 0.4-0.6). Cells were then washed twice and resuspended in sterile PBS and supplemented with 35µM of Histatin-5 (Sigma) to an OD_{600nm} = 0.1±0.01. Cell suspensions were incubated at 30ºC with under agitation (250 rpm) and cell viability was measured at 30 minutes intervals. Each result was compared with the initial cell content of the suspension in order to obtain a survival curve.

3.5 - Spot and Growth Assays

The susceptibility to different test compounds (Table 2) of the parental strains Kchr606, BY4741 and L5U1, and derived deletion mutants, was assessed by comparing their growth in MMB or YPD medium supplemented or not with inhibitory concentrations.

Table 2 - Test Compounds considered for Susceptibility Assays

Compound	Class	Concentrations
Acetic Acid	Weak acid	85 mM; 100 mM
Benzoic Acid	Weak acid	1m M; 1.5 mM
Propionic Acid	Weak acid	10 mM; 20 mM
Amphotericin B	Polyene	0.17 mg/L; 0.19 mg/L; 0.21 mg/L
Mancozeb	Agricultural Fungicide	5 mg/L; 6 mg/L; 7 mg/L

Clotrimazole	Azole	2.5 mg/L; 5 mg/L; 7.5 mg/L
Itraconazole	Azole	40 mg/L; 60 mg/L; 80 mg/L
Ketoconazole	Azole	10 mg/L; 20 mg/L; 30 mg/L
Tioconazole	Azole	0.1 mg/L; 0.3 mg/L; 0.5 mg/L
Fluconazole	Azole	140 mg/L; 160 mg/L; 180 mg/L
Miconazole	Azole	0.2 mg/L; 0.3 mg/L; 0.4 mg/L
Spermine	Polyamine	8.5 mM
Spermidine	Polyamine	10.5 mM
Putrescine	Polyamine	0.017 mM

Cell suspensions used as inocula for spot and growth assays were grown in MMB (or YPD for polyamines) medium until mid-exponential phase (OD_{600nm}=0,5 ± 0.05). For spot assays, cells were diluted in sterile water to obtain suspensions with OD_{600nm}= 0.05 ± 0.005. These and subsequent dilutions (1:5, 1:25) were applied as 4 µL spots onto solid MMB or YPD medium, supplemented with each respective test compound concentrations. Cell suspensions used for growth assays were harvested by filtration and placed in fresh medium supplemented with each respective test compound solution concentrations. Growth rate was monitored until attaining stationary phase, over a period of 80-90 hours.

3.6 - Acetic acid and Spermidine accumulation assays

To analyse the accumulation ratio (intracellular/extracellular concentration) of radiolabeled [^{14}C]-acetic acid in *C. glabrata* Kchr606 and in Kchr606_∆dtr1, cells of the two strains grown in MMB growth medium (at pH 4.0) until mid-exponential phase (OD_{600nm} = 0.5 ± 0.05), harvested by filtration, washed with fresh medium and finally resuspended in 5 mL of this same medium to obtain dense cell suspensions (OD_{600nm} = 0.7 ± 0.05). The same was preformed for the *C. glabrata* Kchr606_∆tpo4 and Kchr606 strains, but since it was a polyamine accumulation assay, YPD growth medium was used. This cell suspensions were incubated for 5 min at 30 °C with agitation (150 rev/min). After that time, 10 µM of [^{14}C]-acetic acid (Amersham Biosciences) were added to the cell suspension together with 65 mM cold acetic acid (at pH 4.0). The intracellular accumulation of radiolabeled acetic acid was followed during 20 minutes by filtering, at adequate intervals, 200 µL of the cell suspension through pre-wetted glass microfibre filters (Whatman GF/C). The filters were washed with ice-cold Tris-Magnesium Sulfate buffer (0.1 M Mes, 41 mM Tris, both from Sigma, adjusted to pH 4.0) and the radioactivity was measured in a Beckman LS 5000TD scintillation counter. Extracellular concentration of [^{14}C]-acetic acid was estimated by measuring the radioactivity of 50 µL of the culture supernatant. Non-specific adsorption of acetic acid to the filters and to the cells was assessed and taken into consideration (less than 5% of the total bound-radioactivity). Intracellular concentration of [^{14}C]-acetic acid was calculated considering the internal cell volume (Vi) of the two strains constant and equal to 2.5 mL (mg dry weight)-1 (Carmelo *et al.*,1997). The same was preformed for the Kchr606_∆tpo4 and

Kchr606 strains, using 0.1 µM of [^{3}H]-spermidine (Amersham Biosciences) added to the cell suspension together with 8.5 mM cold spermidine (Sigma) and the intracellular accumulation of radiolabeled spermidine was followed during 80 minutes.

3.7 - Biofilm quantification Assay

C. glabrata strains were tested for their capacity for biofilm formation recurring to the crystal violet method [117]. For that, *C. glabrata* strains were grown in SDB medium and harvested by centrifugation at mid exponential phase. The cells were incoculated in 96-well polysterene titter plates (Greiner), where previously had been inoculated RPMI medium (pH=4). Each well was inoculated so that the initial OD_{600nm} = 0.05 ± 0.05. Cells were cultivated at 30°C during 24±0.5 hours with mild orbital shaking of 70 rpm. After the incubation time each well was washed three times with 200 µL of deionized water to remove cells not attached to the biofilm matriz. Then 200 µL of a 1% crystal violet (Merk) alcoholic solution was used to stain the biofilm present in each well. Following 15 minutes of incubation, each well was washed with 250 µL of deionized water. The stained biofilm was eluted in 200 µL of 96% (v/v) ethanol and the absorbance of each well was read in a microplate reader at the wavelenght of 590nm (SPECTROstar Nano, BMG Labtech).

3.8 – Subcellular localization of Dtr1 and Tpo4 transporter proteins

C. glabrata cells harboring the pGREG576_MTI_*CgDTR1* plasmid were grown to mid-exponential phase in minimal medium, and then transferred to the same medium containing 1 M CuSO4. At a standard OD_{600nm} of 0.5±0.05, obtained after around 5h of incubation, cells were inspected through fluorescence microscopy. *S. cerevisiae* cells harboring the pGREG576_*CgDTR1* plasmid were also tested considering the same standard OD_{600nm}, obtained after around 5h of incubation with 1% galactose to induce protein expression.

3.9- Statistical Analysis

Statistical analysis of the survival assays results were performed using the Mantel Cox test. For the rest, all data was analyzed with IBM® SPSS® Statistics version 23 by performing oneway ANOVA tests. *P-value*s equal or inferior to 0,05 were considered statistically significant.

4. Results/Discussion

Results of this work are subjected to a confidentially agreement. Consequently they are presented in the confidentiality appendix, which are only available in the confidential version of the thesis.

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