Biofilm formation by the human pathogen *Candida glabrata*: The regulator CgEfg2 and its targets

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Candida glabrata is an opportunistic pathogen capable of forming biofilms, that favor persistent colonization and infection. Understanding of the molecular mechanisms controlling this phenomenon is key to devise improved antifungal strategies. The role of Efg2, encoded by ORF CAGL0M07634g, on the control of C. glabrata biofilm formation was investigated. Results show that Efg2 is involved in the adhesion to biotic and abiotic surfaces. RNA-seq experiments suggest that when cultured under biofilm conditions C. glabrata appear to experience carbon and nitrogen limitation, and hypoxia, which appears to be linked to decreased cellular ergosterol concentrations. Genes related to adhesion and stress and drug resistance were also found up-regulated in biofilm cells. One third of the C. glabrata genes up-regulated in biofilm cells were found to be controlled by Efg2, including many related to the biological functions indicated above. Based on the indications coming from the transcriptomics data, the role of Efg2 in several processes was investigated, showing that Efg2 does not control ergosterol levels in biofilm cells, does not confer azole drug resistance in planktonic cells, but partially controls pseudohyphal differentiation. Efg2 was found to be permanently localized throughout the whole cell, indicating that nuclear accumulation is not required for Efg2 activation. The motives "CGATGS", "CCATTGTY", and "CASAGAA" were predicted as binding sites of Efg2. This work contributed to further the knowledge on biofilm formation in C. glabrata and on the role of Efg2 in the process, and is expected to contribute to the design of improved anti-biofilm therapies.

Key words: *Candida glabrata*, *EFG2*, biofilm formation, RNA-seq.

Introduction

Candida glabrata was initially identified as non-pathogenic, but presently is considered an opportunistic pathogen. *C. glabrata* is currently the second most common cause of Candida infections, that target the bloodstream, oropharynx and urinary tract, being only surpassed by *Candida albicans*. [1][2][3] Many forms of infection caused by Candida species can be associated with the formation of biofilms. Biofilms are 3D aggregates of cells that grow attached to each other and a surface, which can be a biotic surface such as an epithelium or a mucosa, or an inert surface such as a polymer that constitutes a medical device. [4] Medical devices such as catheters represent a serious hazard for patients since they can be reservoirs of microorganisms and entry points for these pathogens. [5]

Infections by *Candida spp*. are particularly hard to treat due to these organisms' characteristics like drug resistance, expression of virulence factors and biofilm formation. [6] Possible causes for the increased resistance to drugs by biofilm-grown cells are the presence of extracellular polymeric substances (EPS) which can act as a physical barrier that prevents the antifungal agent from reaching the cells [7], the expression of efflux pumps [8], and the development of persister cells. [9]

C. glabrata biofilms are organized either in a multilayer structure with cells intimately packed or organized in clusters and its EPS was described as being composed of high amounts of proteins and carbohydrates. [10]

The process of biofilm formation is well described *in vitro* for *C. albicans* and can be divided in four stages The first stage is known as early stage and consists in the adhesion and development of blastospores into discrete microcolonies. The intermediate stage is the second stage during which the Candida biofilm develops as a bilayered structure with a mix of yeasts, germ tubes, and young hyphae. This stage is also characterized by the production of EPS. The maturation stage is the third stage. During this stage, the biofilm develops into a thick EPS layer that surrounds a mesh of yeasts, pseudohyphae, and hyphae. [7] Finally, daughter cells can develop as non-adherent yeast, in a fourth stage called dispersal.[11]

The process of biofilm formation is controlled by several transcription factors (TFs). The Efg1 TF is essential for hyphal differentiation in *C. albicans.* [12] Efg1 also plays an important role in *C. albicans* virulence [13], and biofilm formation. [14] *EFG1* depleted cells also display higher sensitivity to azoles, amphotericin B, and caspofungin. [15] The less studied *C. glabrata* has two predicted *C. albicans EFG1* homologs, encoded by the *CAGL0L01771g* (*EFG1)* and *CAGL0M07634g (EFG2)* open reading frame (ORF), both uncharacterized.[16] [5] *EFG2* will be further analyzed during this work.

Materials and methods

Strains and plasmids. The Saccharomyces cerevisiae BY4741 strain (MATa, Δura3, Δleu2, Δhis3, Δmet15) was obtained from the Euroscarf collection, and used in the present work to construct the plasmids by homologous recombination. The *C. glabrata* strain KUE100 and its derivative single deletion mutants KUE100_Δcgefg1, and KUE100_Δcgefg2 were kindly provided by Hiroji Chibana, from the Medical Mycology Research Center (MMRC), Chiba University, Chiba, Japan. The *C. glabrata* strain L5U1 (Δcgura3, Δcgleu2) was kindly provided by John Bennett from the National Institute of Allergy and Infectious Diseases, NIH, Bethesda, USA. The C. glabrata strain CBS138, whose genome sequence was released in 2004, was used in this study for gene amplification purposes. The VK2/E6E7 human epithelium cell line (ATCC® CRL-2616™) was used for adhesion assays. This cell line is derived from the vaginal mucosa of healthy premenopausal female submit to vaginal repair surgery, and immortalized with human papillomavirus 16/E6E7. The plasmid pGREG576 was obtained from the Drag & Drop collection. [17]

Biofilm quantification assays. Biofilms formed by *C. glabrata* cells were quantified using the Prestoblue method[18] For the Prestoblue method, *C. glabrata* cells were cultured at 30ºC, overnight, with

orbital agitation (250 rpm), in MMG-U liquid medium. MMG-U medium was composed by minimal medium [containing per liter 2.7 g $(NH_4)_2SO_4$ (Merck), 1.7 g yeast nitrogen base without amino acids or $(NH_4)_2SO_4$ (Difco), and 20 g of glucose (Merck)], supplemented with 60 mg/L Leucine. After overnight cultivation, the OD_{600} of the cultures was measured to calculate the necessary volume to prepare a fresh culture with a final OD_{600} of 0.6. When adequate, these freshly prepared cultures were supplemented with 200 µM $CuSO_4$ (Sigma) to induce the promoter sequence upstream the *EFG2* gene cloned in pGreg576. Once the cultures reached the desired OD_{600}, a suspension was prepared with an OD_{600} of 0.1 and was supplemented with 400 µM of $CuSO_4$ solution. The cell suspension was then used to inoculate a 96-well polystyrene titter plate (Greiner) previously filled with twice concentrated SDB liquid medium. The twice concentrated SDB medium, equilibrated at pH 5.6, contained (per liter): 80 g glucose (Merck) and 20 g meat peptone (Merck). The pH of 5.6 was obtained using a HCl 1M solution. All wells of the microplate were filled with 100 µL medium, and the first 10 columns were inoculated with 100 µL of cellular suspension (first four rows strain L5U1+VV and last four rows with strain L5U1+Efg2) while the last two rows were inoculated with sterile water. The microwell plates were sealed with a membrane (Greiner Bio-One) and incubated at 30°C, with gentle orbital agitation (70 rpm) for 15h. After this time the growth medium from the biofilm-coated wells of microtiters plates was removed, and the biofilm formed was washed two times with 100 µl of phosphate buffered saline (PBS) at pH 7.4 to remove cells that were not attached to the formed biofilm. PBS contained per liter: 8 g NaCl (Panreac), 0.2 g KCl (Panreac), 1.81 g $NaH_2PO_4.H_2O$ (Merck), and 0.24 g KH_2PO_4 (Panreac)**.** The pH was equilibrated to 7.4 using a HCl 1M solution. After the washing step, 100 µL of 1:10 Prestoblue (ThermoFisher Scientific) in SDB solution was added to each well to stain the formed biofilm. This was done in darkness due to the photosensitivity of Prestoblue. The plate was covered with foil and was incubated at 37ºC for 30 minutes. The absorbance of the resulting solution was measured in a microplate reader (SPECTROstar Nano, BMG LabTech) at 570 nm and 600 nm. The results were estimated by subtracting the absorbance at 600 nm from those measured at 570 to account for the dead cells. The values obtained from control columns were then subtracted from the inoculated ones to minimize background interference.

Epithelial adhesion assay. The adhesion to the epithelium by *C. glabrata* strains was assessed by counting the number of colonies formed after a 30 minutes incubation with a 12,5 OD_{600} yeast suspension in PBS with human vaginal epithelium. These C. glabrata suspensions were prepared as follows: A colony of each strain was transferred from an Yeast extract –Peptone–Dextrose medium (YPD) plate and used to inoculate 25mL of YPD medium. These suspensions were incubated at 30ºC and 250 rpm for 8 hours. After that time the OD_{600} of the suspensions were measured, and adequate volumes were used to prepare new cultures of 250 mL of YPD with an OD_{600} of 0.05. The new suspensions were incubated overnight at 30ºC and 250 rpm. After incubation, OD_{600} was measured again to calculate the volume necessary to prepare yeast cellular suspensions in PBS with an OD_{600} of 12.5. The total colony forming units (CFUs) present in the PBS suspensions prepared were estimated by plating serial 1:10 dilutions on YPD medium. Vaginal epithelium was placed in wells of a 24 well plate. On the top of each well, containing epithelium cells, 10 µL of PBS suspension were pipetted. The plate was centrifuged for 1 minute at 1000 rpm in a Eppendorf 5804 centrifuge and was incubated for 30 minutes at 37ºC in a 5% CO_2 atmosphere. After incubation, the medium was removed, and three washes with 500 µL of PBS were performed. The washes were followed by 15 minutes incubation with Triton x100 at 0.5%. The wells were then scrapped, and the media was collected. Serial 1:10 dilutions were then made and plated on YPD plates. After incubation at 30ºC for 48h, the colonies were counted.

S. cerevisiae and C. glabrata transformation. Cultures of *S. cerevisiae* BY4741 and *C. glabrata* L5U1 were carried out at 30ºC, 250 rpm in YPD, containing, per liter,20 g glucose (Merck), 20 g bacterial peptone (Dickson) and 10 g of yeast extract (HIMEDIA). Once the cultures reached an OD_{600} of 0.4 the cells were harvested, and the transformation reactions were performed using the Alkali-Cation Yeast Transformation Kit (MP Biomedicals) according to manufacturer's instructions. Transformant cells were plated in appropriate agarized media

E. coli transformation. An aliquot (150µL) of *E.coli* competent cells stored at -80ºC were slowly defrosted on ice. After the cells were completely defrosted, 50 µL of TCM solution and 15µL of plasmid suspension were added to the Eppendorf tube. The TCM solution contained 10 mM $CaCl_2$, 10 mM $MgCl_2$, and 10 mM Tris HCL at pH 7.5. The cells were then incubated on ice for 15 minutes, heat shocked at 42ºC for 3 minutes, and incubated again on ice for 5 minutes. After these thermal treatments, 800 µL of liquid Luria broth (LB) were added to the Eppendorf tube containing the cell suspension. The cells were incubated for one hour, at 37ºC and 250 rpm. The liquid LB medium was composed per liter by: 25g LB broth (Sigma). The cells were then harvested by centrifugation. Cell pellets were resuspended and plated on pre-heated LB solid medium [containing 20 g agar (iberagar)] supplemented with 150 µg/mL ampicillin(Sigma) plates.

Cloning the MT-I promoter sequence upstream the EFG2 gene. The pGREG576 plasmid was used to clone the MT-I promoter sequence upstream the *EFG2* gene (ORF *CAGL0M07634g*). This gene was cloned after amplification by PCR.

RNA-sequencing analysis. RNA-sequencing (RNA-seq) analysis was carried out to identify which genes were differently expressed when comparing planktonic or biofilm grown cells of *C. glabrata*. The same methodology was performed to identify genes differently expressed between *C. glabrata* KUE100 and its deletion mutant Δefg2. For these purposes, planktonic cells were obtained from cultures grown in SDB medium, at 30°C, with orbital agitation (250 rpm), when reaching an OD_{600} of 1. Biofilm grown cells were obtained from 24h grown cultures with SDB medium and gently agitated (30 rpm) at 30ºC, carried out in square plates (Greiner, 120x120x17mm), with an initial OD_{600} of 0.05. Total RNA of cells cultivated as described above was isolated using Ambion Ribopure-Yeast RNA kit, according to manufacturer's instructions. Strand specific RNA-seq library preparation and sequencing was carried out as a paid service by the NGS core from Oklahoma Medical Research Foundation, Oklahoma City, Oklahoma, USA. Paired-end reads (Illumina HiSeq 3000 PE150, 2x150 bp, 2 GB clean data) were obtained from wild type *C. glabrata* KUE100 and correspondent deletion mutant strain KUE100_Δcgefg2 (ORF CAGL0M07634g). Two replicates of each sample were obtained from three independent RNA isolations, subsequently pooled together. Sample reads were trimmed using Skewer [19] and aligned to the *C. glabrata* CBS138 reference genome, obtained from the Candida Genome Database (CGD) (http://www.candidagenome.org/), using TopHat [20]. HTSeq [21] was used to count mapped reads per gene. Differentially expressed genes were identified using DESeq2 [22] with an adjusted p-value threshold of 0.01 and a log2 fold change threshold of -1.0 and 1.0. Default parameters in DESeq2 were used. Candida albicans and Saccharomyces cerevisiae homologs were obtained from the Candida Genome Database and Saccharomyces Genome Database (SGD) (https://www.yeastgenome.org/), respectively. Manual clustering of the up- and down-regulated genes was carried out, based on the description of the C. glabrata genes found on the Candida Genome Database (CGD) (http://www.candidagenome.org/, last accessed at 12/05/2018). Uncharacterized genes were clustered based on the description of ortholog genes in S. cerevisiae or in C. albicans, according to the

Saccharomyces Genome Database (SGD) (https://www.yeastgenome.org/, last accessed at 12/05/2018) or in CGD, respectively.

Antifungal susceptibility assays. This procedure was performed according to the EUCAST norms. [23]

Quantification of ergosterol in C. glabrata strains. These experiments were performed as described by Santos et al. [24]

Pseudohyphal differentiation assays. To stimulate pseudohyphal differentiation in *C. glabrata* cells the tested strains, KUE100 and Δefg2, were cultured in YPD medium overnight. New cell batches were then prepared in YPD medium supplemented with 125 µL of isoamyl alcohol solution, with an initial OD_{600} of 0.1. The new cell batches were grown for 48h with orbital agitation (250 rpm) at 30ºC. After the stipulated time 7 µL were collected from each batch and were observed by microscopy with a Zeiss Axioplan microscope (Carl Zeiss MicroImaging). Images were captured using a Axiocam 503 Color (Zeiss). Percentage of pseudohyphal differentiation was computed manually.

Efg2 subcellular localization assessment. The subcellular localization of the Efg2 TF was determined based on the observation of L5U1 *C. glabrata* cells transformed with the pGREG576+MT-I+*EFG2* plasmid. These cells express the Efg2+GFP fusion protein, whose localization may be determined using fluorescence microscopy. *C. glabrata* cell suspensions were prepared in minimal medium supplemented with 60 mg/L leucine (MMG-U), and were incubated overnight at 30ºC, and 250 rpm. For planktonic grown cells a second culture was prepared from the first cell culture to have an OD_{600} of 0.02. This culture was supplemented with 50 µM $CuSO_4$ (Sigma), to induce protein overexpression. The culture was kept at 30ºC and 250 rpm until significant fluorescence was observed under the microscope. For biofilm grown cells a second culture was prepared from the first culture to have an OD_{600} at the time of the transfer to the square plates of 0.6. This second culture was supplemented with 200 µM of $CuSo_4$ (Sigma). When the intended OD was reached a 40 mL suspension was prepared in SDB growth medium with an OD_{600} of 0.05 in a square plate. The cell suspensions were incubated for 6h or 24h at 30ºC and 30 rpm. After the established time the supernatant was discarded, and the attached cells were scraped using a spatula. For observation under the microscope 7µL were collected from each strain and each growth type. The distribution of Efg2+GFP fusion protein in *C. glabrata* living cells was detected by fluorescence microscopy in a Zeiss Axioplan microscope (Carl Zeiss MicroImaging), using excitation and emission wavelength of 395 and 509 nm, respectively. Fluorescence images were captured using a Axiocam 503 Color (Zeiss).

Results and discussion

Efg2 is determinant for biofilm formation. In the present work, the eventual role played by the uncharacterized *C. glabrata* Efg1 and Efg2 transcription factors, encoded by ORFs *CAGL0L01771g* and *CAGL0M07634g*, respectively, on biofilm formation was investigated, given their high homology to the Efg1 transcription factor from *Candida albicans* [14], implicated in this phenomenon. Preliminary unpublished results (not shown) suggest that *CgEFG2* plays a role in biofilm formation process unlike *CgEFG1*. Given this initial result, all studies were focused on Efg2.

To confirm the importance of Efg2 in biofilm formation, the effect of over-expressing *EFG2* in the level of biofilm formation in a polystyrene surface was assessed. Wt strain L5U1 overexpressing *EFG2*, from the pGreg576_MT-I_*EFG2* plasmid, was compared to the same cells harboring the pGreg576 cloning vector (L5U1+VV) in terms of biofilm formation (Figure1).

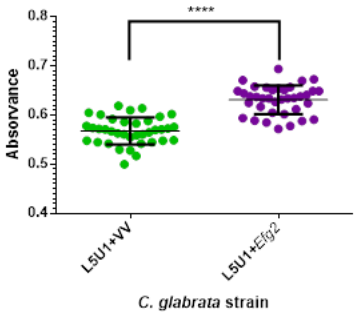

*Figure 1 - Role of Efg2 on C. glabrata ability to form biofilm on a polystyrene surface. Prestoblue was used to stain the C. glabrata wt (L5U1+vv) and the mutant strain overexpressing EFG2 (L5U1+EFG2). Each dot results from a single absorbance measurement. A total of 37measurements were performed for each strain. **** indicates a P-value<0.0001.*

These results are consistent with the notion that Efg2 is a key player in *C. glabrata* biofilm formation, as the level of biofilm formation increases upon moderate overexpression of the *EFG2* gene, when compared to the parental strain, harboring the empty vector pGreg576.

To understand whether Efg2 also plays a role in biofilm formation in epithelial cell surfaces, the effect of *EFG2* deletion in *C. glabrata* adhesion to VK2/E6E7 human vaginal epithelial cells was evaluated (Figure 2).

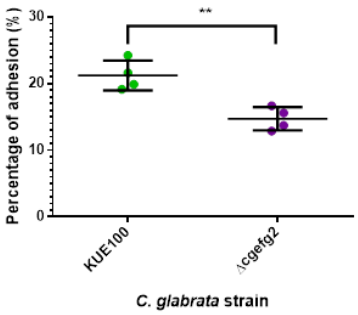

*Figure 2 - Average percentage of adhesion of C. glabrata strains KUE100 and its deletion mutant Δefg2 to the vaginal epithelium cells. The standard deviations are indicated, as well as the average percentage for each trial. **indicates a P-value<0.01.*

The results obtained show that *EFG2* deletion significantly decreases *C. glabrata* adhesion, leading to 30% lower concentration of adhering cells. All these results suggest that the *EFG2* gene plays not only a part in the adhesion process of *C. glabrata* to inert surfaces but also to mucosal surfaces.

Transcriptomics analysis of the role of EFG2 in biofilm formation. To assess the role of *EFG2* in biofilm formation, the effect of its deletion on global gene expression was evaluated through RNA-sequencing, focusing especially in changes occurring from planktonic growth to biofilm formation. The obtained results are detailed and discussed below, starting with the analysis of gene expression changes observed in cells cultivated as biofilm for 24h, when compared to planktonic cultivation, and followed by the analysis of the genes whose expression was found to be controlled by *EFG2* in biofilm cells.

1. Global transcriptional remodeling occurring during biofilm formation in C. glabrata. To unveil the patterns of gene expression associated with biofilm cells, when compared to planktonic growth in C. glabrata, an RNA-seq analysis was performed, and the results were afterwards biologically interpreted. The transcriptome of exponentially growing planktonic cells, grown in SDB medium and harvested with an OD_{600} of 1.0, was compared to that of biofilm cells, cultivated in the same medium for 24h in polystyrene dishes. The transcriptomes from wilt-type cells grown

planktonically or as biofilms were compared. This analysis revealed a total of 3072 genes with an altered expression, which corresponds to around half of the C. glabrata genome, which contains to this date 5293 identified ORFs. The most represented biological functions among genes up-regulated genes in biofilm cells are "RNA metabolism and translation" (26.2%), "unknown function" (21.6%), "Carbon and energy metabolism" (6.7%), "Cell cycle" (5.9%) and "Response to stress" (5.1%). The most represented biological functions among genes down-regulated genes in biofilm cells are "RNA metabolism and translation" (13.5%), "unknown function" (12.9%), "Cell cycle"(12.2%), "Intracellular trafficking" (9.6%), "DNA metabolism and repair" (9.0%), "Carbon and energy metabolism" (7.4%) and "Protein metabolism"(7.4%). One of the first transcriptomics studies on biofilm formation by *C. albicans* was carried out by García-Sánchez et al. [25]. In their work, the transcriptome of 72h *C. albicans* biofilm cells was compared to the transcriptome of planktonic grown cells. The authors used macroarrays with 2002 probes which represents approximately one third of the *C. albicans* genome. A group of 325 genes was found to be differently expressed according to the growth conditions of the cells. Those genes were grouped into 25 functional groups according to their homology to *S. cerevisiae* genes. The functional groups that were enriched in this analysis were "amino acid metabolism", "nucleotide metabolism", "lipid, fatty acid, and isoprenoid metabolism", "transcription", "protein synthesis", "Control of cellular organization", "Subcellular localization" and "Unclassified".

The high representation of the "RNA metabolism and translation" group in both up- and down-regulated subgroups of differently expressed genes found in our RNA-seq analysis of *C. glabrata* cells grown under biofilm and planktonic conditions seems to be consistent with the observations of García-Sánchez et al..[25] In their analysis a high number of genes differently expressed were related to mRNA and rRNA processing and stability, as well as ribosomal proteins and aminoacyl-tRNA synthases that are included in categories "protein synthesis" and "transcription". [25] Opposite to these authors we observed as major categories of differently expressed genes "carbon and energy metabolism", "cell cycle", "response to stress", "Intracellular trafficking" and "DNA metabolism and repair". These differences might result from the fact that in this analysis the number of probes used only represents a third of the *C. albicans* genome which might not give a very representative view of the changes occurring in the expression of the genes as well as some differences between species.

Nett et al. also studied the transcriptome of *in vivo* biofilms formed by *C. albicans* at different developmental stages: intermediate (12h), and mature (24h). Since the biofilms from *C. glabrata* cells are fully mature at 24h when grown *in vitro*, our comparison will focus on the 24h biofilm of *C. albicans* cells grown *in vivo*. From the 6354 ORFs analyzed by Net et al., 1034 genes were differently expressed in 24h biofilms when compared to planktonic grown cells. From these genes, 523 were up-regulated and 511 were down-regulated. In their work the categories of genes that are more up-regulated in 24h biofilms are "unknown", "transcription/protein", "energy metabolism", "carbohydrates", "DNA/cell cycle", and "transport". The up-regulation of genes related to RNA metabolism in our analysis goes into agreement to the results of Nett et al. [26] even though they are placed in the same functional group as protein. In both analysis genes related to cellular duplication and carbon metabolism appear up-regulated. Besides energy production, another possible reason for the up-regulation of carbon metabolism is the synthesis of the EPS that makes up the matrix where the biofilm cells are involved, even though this process should be more active in the intermediate stage of development of the biofilm. Unlike what is observed in Nett et al. [26] analysis, our RNA-seq analysis shows that genes related to response to stress represent one of the functional groups most present in the up-regulated sub-set. This difference might be related to the fact that the thickness of the EPS of *C. albicans* cells in biofilms is greater than the one of *C. glabrata*. This lower thickness leaves the *C. glabrata* biofilms more subjected to the environment conditions, increasing the necessity to activate stress responsive mechanisms.

In the transcriptome analysis of Nett et al. [26] the functional groups that appear most represented among the down-regulated set of genes are "unknown", "transcription/protein", "DNA/cell cycle", "transport", "energy metabolism". These observations are in agreement with ours. A result that jumps to sight in our analysis as well as the ones performed by García-Sánchez et al. [25] and Nett et al. is the high number of genes with unknown functions. This might be justified with either lack of homology to *S. cerevisiae* or *C. albicans* genes or the very high amount of genes uncharacterized in *C. albicans* (70%) (http://www.candidagenome.org/cache/C_albicans_SC5314_genomeSnapshot.html) and, even more, in *C. glabrata* (95%) (http://www.candidagenome.org/cache/C_glabrata_CBS138_genomeSnapshot.html).

Selected functional groups and their relation to biofilm formation are discussed in more detail bellow.

1.1. Multiple stress response pathways are up-regulated during biofilm formation. Within the group of stress response genes, 80 were found to be up-regulated, while 46 were found to be down-regulated during biofilm formation. Among the up-regulated genes, 14 encode chaperones predicted to be involved in the response to heat shock and other protein denaturing conditions, and 11 encode oxidative stress response proteins. Many of the up-regulated genes also encode stress responsive transcription factors, involved in the general stress response, namely *MSN1* and *MSN4*, in oxidative and metal stress response, *AP1*, *CAD1*, *YAP6* and *YAP7*, in acid/alkaline stress response, *RIM101*, *HAA1*, *WAR1*, in the unfolded protein response, *HAC1*, and in osmotic stress response, *HOT1*, among others. Altogether, biofilm cells appear to either sense a stressing environment or be highly prepared to face one. In the work of Yeater et al. the changes in gene expression throughout the process of biofilm development were analyzed in *C. albicans* cells. They also report changes in the expression of genes related to several environmental stresses. [27]

1.2. Multiple drug resistance genes are up-regulated during biofilm formation. Biofilms are associated to higher resistance to drugs. Although this is not a result of a single factor, it can be linked to the overexpression of genes encoding efflux pumps and the reduction of antifungal accumulation within the cell. [28] Interestingly, among the up-regulated genes in biofilm cells are a number of genes encoding multidrug resistance (MDR) transporters from the Major Facilitator Superfamily (MFS) However, the *CDR1* gene, encoding the major ATP binding cassete (ABC) drug efflux pump involved in azole resistance, was found to be down-regulated in 24h biofilm cells. This observation goes into agreement with works that indicate that the role of Cdr1 is more relevant in the early and intermediate stages of biofilm development. [8][29]. In the work of Nett et al. the up-regulation of drug resistance-related genes was also observed throughout the developmental stages of *C. albicans* biofilms *in vivo*. [26] In the work of Yeater et al. differently expressed genes that encode efflux pumps is also reported in biofilm grown *C. albicans* cells. The differences in expression are however attributed to specific combinations of strains and substrates. This is consistent with the idea that a multifactorial mechanism dependent on the substrate and specific strain is responsible for the drug resistance displayed by biofilms, particularly those constituted by *C. albicans*. [27] Unlike what is reported in the two mentioned articles, García-Sánchez et al. have found no significant differences in expression of the genes *CDR1*, *CDR2*, *CDR3*, *CDR4*, and *MDR1*. This might be due to the fact that the work of García-Sánchez was performed in 72h biofilms while the other works focus on biofilms

in earlier stages of development, therefore having different patterns of gene expression. [25]

Besides the up-regulation of the expression of genes coding for efflux pumps and transporters, there are other possible sources of increase in drug resistance displayed by cells cultured under biofilm conditions. The other attributed causes are the presence of an EPS layer surrounding the cells, which protects them from some external unpleasant conditions, and the existence of persister cells, which are cells that are able to resist the action of antifungals due to a general slowdown of their metabolism. [7][9] Nevertheless, the reason why biofilm cells display increased expression of multidrug transporters, in the absence of drugs, is still to be understood.

1.3. Adhesion-related genes are up-regulated during biofilm formation. Adhesion to surfaces and between cells is the first step of biofilm formation, being required to maintain the mature biofilm [30][31]. Thus, the observed up-regulation of genes related to adhesion was clearly expected. These include genes encoding cluster I, II, III and IV adhesins. In the works of Nett et al. [26], García-Sánchez [25], and Yeater et al. [27] genes of the ALS family of *C. albicans* were found to be differently expressed in cells under biofilm growth conditions. Interestingly, ORFs *CAGL0M01716g* and *CAGL0M07634g*, predicted to encoded biofilm-related transcription factors, were also found to be up-regulated in biofilm cells. Each of these ORFs encodes one of the two predicted orthologs of *C. albicans* Tec1 and Efg1 transcription factors, respectively, which play a massive role in biofilm formation in this pathogenic yeast [32]. It is interesting to point out that within the two predicted *C. albicans* Efg1 homologs in *C. glabrata*, Efg1 and Efg2, it is the Efg2 encoding ORF that was found to be up-regulated in biofilm cells, which is consistent with the role played by the *C. glabrata* Efg2 transcription factor, but not by Efg1, in biofilm formation, as described in this work.

1.4. Virulence-related genes are up-regulated during biofilm formation. A relatively small number of virulence related genes was found to change in expression in biofilm cells, when compared to planktonic cultivation. However, the whole list is composed of YPS genes, encoding a family of glycosylphosphatidylinositol-linked aspartyl proteases, shown to be required for full virulence in mouse models [33]. In *S. cerevisiae* the yapsin gene family is composed by five (GPI)-linked aspartyl proteases that cleave C-terminal domains of peptides in *in vitro and in vivo* conditions. These genes are usually expressed during cell-remodeling processes. Furthermore *S. cerevisiae* deletion mutants for *YPS* genes have been shown to have reduced amounts of β-1,3 and β-1,6 glucans in their cell wall constitution. [34] The YPS gene family plays a key role in cell wall remodeling by removing (GPI)-cell wall proteins. The expression of these genes seems to indicate either a necessity to remove certain (GPI)-CWPs to replace them with others more suited to a new environment or might be a protection mechanism developed by *C. glabrata* in which targets of the innate or adaptive immune system are removed from the cell surface. [33]

1.5. Changes in amino acid and nitrogen metabolism gene expression during biofilm formation. Among the genes whose expression changes in biofilm cells, when compared to those growing planktonically, we found 127 related to amino acid and nitrogen metabolism. Overall, the synthesis of histidine, phenylalanine, glutamate and arginine appears to be up-regulated. The biosynthesis of sulfur amino acids, cysteine and methionine, were found to be down-regulated under our experimental conditions, as well as the hydrophobic apolar amino acids valine, leucine, isoleucine and glycine. Interestingly, there appears to be an up-regulation of deamination reactions leading to α-oxoacids intermediates, as is the case of the reactions that convert serine into pyruvate, glutamine into glutamate, and threonine into 2-oxobutanoate. Simultaneously there seems to be a down-regulation of genes involved in the conversion of α-oxoacids into amino acids, like valine, leucine, isoleucine, proline, and glycine. In addition, several intermediate reactions involved in the synthesis of these amino acids are up-regulated, suggesting that the pathways are partially activated. This suggests that *C. glabrata* cells undergoing biofilm growth are using at least some of these amino acids to generate the corresponding α-oxoacids. This might represent a metabolic shift favoring the energetic metabolism instead of the amino acid biosynthesis pathway, suggesting that the cells might be experiencing nitrogen starvation. This is probably due to their high degree of adhesion to each other and due to the fact they are embedded in a EPS matrix which causes the more internal cells to get reduced access to nutrients, namely nitrogen sources.

In the work by García-Sánchez et al. *C. albicans* cells from 72h biofilms display an overexpression of genes involved in the synthesis of aromatic and sulfur amino acids. [25] In the work performed by Nett et al. with *C. albicans* cells grown under *in vivo* biofilm conditions, the cells from biofilms in more mature states of development instead of expressing differently genes that codify enzymes involved in the synthesis of sulfur amino acids, have the expression of genes encoding amino acid permeases up-regulated. [26]. Within the nitrogen metabolism group the major functions present are transport of polyamine and ammonium. Altogether, our results from amino acid metabolism and nitrogen metabolism strongly suggest that *C. glabrata* in mature biofilms experience nitrogen limitation.

1.6. Changes in carbon and energy metabolism gene expression during biofilm formation. To further investigate the metabolic change being experienced by *C. glabrata* cells when cultured under biofilm conditions when comparing with cells grown under planktonic conditions, the 216 genes grouped under carbon and energy metabolism category were studied using the Kegg mapper tool (http://www.kegg.jp/kegg/mapper.html). The distribution of up- and down-regulated genes from the carbon and energy metabolism group shows that there is a down-regulation of the glycolytic pathway and instead there is an up-regulation of the glyoxylate cycle which might indicate that biofilm cells are using alternative sources of carbon to glucose. A possible alternative source of carbon are 2 carbon molecule sources that come from β-oxidation pathway. In the work Nett et al. with *C. glabrata in vivo* biofilms, there is also an up-regulation of the glyoxylate cycle. [26]

To further assess whether the hypothesis of carbon sources being redirected from β-oxidation would be plausible the Kegg mapper tool was used with the Lipid metabolism group of genes. Results show an up-regulation of the pathways that lead to the degradation of fatty-acids. Simultaneously there is a down-regulation of the synthesis of long chain fatty-acids. This favoring of the β-oxidation together with the up-regulation of genes involved in the glyoxylate cycle seems to agree with the hypothesis that *C. glabrata* cells under biofilm growth conditions might be using alternative carbon sources. In each cycle of β-oxidation there is the formation of a molecule of acetyl-CoA. This molecule can then enter the glyoxylate cycle and be used as a carbon and energy source. In *S. cerevisiae* and in *C. albicans* β-oxidation occurs inside the peroxisomes, it is therefore likely that peroxisomes play a key role in β-oxidation in *C. glabrata*. [35][36] In our RNA-seq data there are 18 genes grouped as "peroxisome biogenesis and organization. From these 18 genes, 13 are up-regulated. All this is consistent to an up-regulation of the β-oxidation pathway in order to use alternative sources of carbon to glucose. Even though in our data set there are no evidences that the gluconeogenesis is up-regulated, the interception of the up-regulation of the β-oxidation and the glyoxylate cycle is indicative of an environment poor in nutrients.

1.7. Changes in ergosterol gene expression during biofilm formation. As discussed in the previous section our RNA-seq data suggests that *C. glabrata* cells undergoing biofilm growth up-regulate genes that encode enzymes involved in β-oxidation pathway. Furthermore the biosynthesis of long-chain fatty acids is down-regulated in our experimental conditions.

To further study the effects of *C. glabrata* biofilm growth on the metabolism of lipids the Kegg mapper tool was used to evaluate the changes at the level of ergosterol metabolism. Among the 30 "ergosterol biosynthesis" genes whose expression was seen to change in biofilm cells, when compared to planktonic cells, 18 are up-regulated. Our results suggest that even though the final steps leading to ergosterol biosynthesis are repressed, the intermediates might be accumulating in biofilm cells. Other alternative explanation would be that in *C. glabrata* the up-regulation of genes involved in the process of ergosterol biosynthesis happens at an earlier stage than the one measure in this work. Nonetheless the role of these intermediates remains unexplained.

1.8. Changes in cell wall metabolism gene expression during biofilm formation. Changes in expression of genes that are involved in the cell wall organization can be relevant for the understanding of the biofilm formation process. During biofilm development the cells are attached to each-other and have to interact with each-other in order to function as a colony. To gain further clues on changes that occur in the expression of genes involved in cell wall organization when cells undergo biofilm growth the Kegg mapper tool was used (http://www.kegg.jp/kegg/mapper.html). All genes involved in GPI-anchor biosynthesis were found to be down-regulated, suggesting that GPI-synthesis is repressed in *C. glabrata* cells at 24h. We speculate that this down regulation of GPI-anchors might be related to the dispersal phase of biofilm formation. In the work of Nett et al. from the genes that were found to be differently expressed at 12h and 24h *C. albicans* biofilm the only gene identified as playing a part in GPI-anchor biosynthesis is *CWH43*, which was found to be down-regulated. [26] The ortholog of this gene in *C. glabrata*, *CWH43*, was not found to be differently expressed in our data set.. Opposite to our observations, Yeater et al. found several genes encoding GPI-linked cell wall proteins up-regulated in *C. albicans* cells from biofilms after 6h and 12h hours. [27] These results were obtained from biofilms at earlier stages of development compared to the stage of development of the biofilm cells used in the present work.

2. Role of Efg2 in biofilm formation. The role of the transcription factor Efg2 in biofilm formation was evaluated based on the comparison of the transcriptome-wide changes occurring upon 24h of biofilm formation in wild-type cells and Δcgefg2 mutant cells. Overall, *EFG2* deletion was found to affect the expression of 863 genes, in planktonic growing cells, and 1162 genes in biofilm cells. Considering only the Efg2-up-regulated genes, 258 were found to be under the control of Efg2 during planktonic cultivation, while 648 were found to be regulated by Efg2 upon 24h of biofilm formation. To evaluate the contribution of Efg2 to the transcriptional remodelling taking place during biofilm formation, the Venn diagram depicted in Figure 3 was constructed. Efg2 was found to positively regulate 466 out of the 1567 genes found to be up-regulated in biofilm cells, which corresponds to roughly controlling 1/3 of the transcriptome-wide changes registered during this process. To gain understanding of what are the biological processes affected by Efg2, the 1162 genes that were found to be differentially expressed in wild-type versus Δefg2 strains in 24h biofilm cells were grouped according to their functional category, and were divided in up-regulated and down-regulated by the Efg2 transcription factor. The most represented biological functions among the Efg2 up-regulated genes in biofilm cells are "unknown" (30.86%), "Protein metabolism" (8.49%), "Response to stress" (7.56%), "Carbon and Energy metabolism" (6.79%) and "Lipid metabolism" (6.17%).

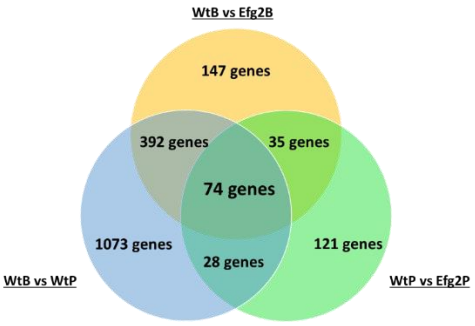

Figure 3 - Venn Diagram illustrating the interception between the up-regulated genes of the three transcriptomic analysis.

The most represented biological functions among the Efg2 down-regulated genes in biofilm cells are "RNA metabolism and ribosomal biogenesis" (13.42%), "Unknown" (12.45%), "Cell cycle" (11.28%), "Carbon and energy metabolism" (10.31%) and "Protein metabolism" (7.98%).

2.1. Efg2 controls the expression of genes involved in stress response. The second biggest group of Efg2 target genes activated in biofilm cells is the one containing 49 genes related to stress response. Among the Efg2 target genes it is possible to find many related to oxidative stress, a few chaperone encoding genes, , and quite a few stress related transcription factors, including those involved in the general stress response, namely *MSN1* and *MSN4*, in oxidative and metal stress response, *CAD1*, in acid/alkaline stress response, *RIM101*, and in the unfolded protein response, *HAC1*, among others. Altogether, Efg2 appears to control several aspects of the stress response activation occurring in biofilm cells. It would be interesting to check whether this transcription factor confers specifically resistance to any of these stresses.

Upon consultation of the PathoYeastract database (http://pathoyeastract.org/), *C. albicans* Efg1 was found to regulate *C2_05860C*, which is a putative transcription repressor induced by oxidative stress, *CAS5*, which is a TF involved in cell wall stress response, *C2_03530W*, that plays a role in the apoptotic process, *YIM1*, whose *S. cerevisiae* ortholog is involved in cellular response to DNA damage stimulus, *YHB1*, that acts in nitric oxide detoxification, *RIM101*, which is a TF involved in alkaline pH response; *YCP4*, which is involved in oxidative stress protection, *SSU1*, which is involved in the efflux of sulfites, *PDE1*, that mediates cAMP signaling in response to intracellular acidification, and *GAC1*, whose orthologs have heat shock protein binding. Interestingly, all these genes have their orthologs down-regulated in the absence of *EFG2* in *C. glabrata* cells cultured under biofilm conditions.Thus, there seems to be a significant overlap of the function of these orthologous transcription factors in the control of stress response in biofilm cells.

2.2. Efg2 controls the expression of genes involved in multidrug resistance. Within the set of genes that are positively regulated by Efg2 there is a subset that is involved in the process of drug resistance. This subset is composed by 13 genes, including those encoding drug transporters of the MFS, and of the ABC superfamilies. This suggests that under biofilm growth conditions Efg2 might be involved in an increase of resistance to azoles by C. glabrata cells. However, it is necessary to point out that C. glabrata Efg2 role in the control multidrug resistance transporter expression does not appear to be shared by C. albicans Efg1. From this group of genes only the C. albicans ortholog of C. glabrata CAGL0M05445g, C1_10140C, is described as being up-regulated by Efg1 in cells cultured under biofilm conditions. C1_10140C codifies for a protein of unknown function, however its S. cerevisiae

ortholog Cos111 confers resistance to the antifungal drug ciclopirox olamine.

2.3. Efg2 controls the expression of adhesion-related genes. Efg2 was found to control the expression of 7 predicted adhesins, including cluster I adhesins encoded by the *EPA9* and *EPA10* genes. Genes *AED1*, *AED2*, and *AWP13*, encoding cluster III adhesins regulated by Efg2 were also found to be up-regulated in biofilm cells, when compared to planktonic cultivation. ORF CAGL0M01716g, predicted to encode a Tec1-like biofilm-related transcription factor, was found to be under the control of Efg2, besides being up-regulated in biofilm cells. This may indicate that, similar to what has been registered in *C. albicans* [32], Efg2 and Tec1 may constitute two key players in the complex regulatory network controlling biofilm formation.

In *C. albicans* Efg1 has also been described to up-regulate some adhesin coding genes such as *ALS1*, *ALS3*, *ALS4*, *ALS6*, *ALS7*, and *ALS9*. This goes into agreement with our results in which Efg2 plays an important role in adhesion to both epithelium and polystyrene in *C. glabrata*.

2.4. Efg2 controls the expression of genes involved in amino acid and nitrogen metabolism. Among genes up-regulated by Efg2 in *C. glabrata* cells cultured under biofilm growth conditions there are some that are involved in the synthesis of amino acids. These genes are involved in the pathway that leads to the biosynthesis of citroline from both glutamine and carbonyl phosphate. They also play a role in the biosynthesis of histidine, lysine and leucine. By using the Kegg mapper tool (http://www.kegg.jp/kegg/mapper.html) twelve Efg2 target genes were found to be involved in the amino acids biosynthesis pathway [*ARG8*, *CAGL0F06501g (CaECM42)*, *CAGL0F06875g (CaLYS1)*, *CAGL0G06732g (ScLEU9)*, *CAGL0G09691g (ScSER1)*, *CAGL0I09009g (ScHIS2)*, *CAGL0I10791g* (which does not have a known ortholog in *C. albicans* or in *S. cerevisiae)*, *CAGL0J03124g* (*CaARG5,6)*, *CAGL0K08580g (CaAAT1)*, *GLT1*, *CAGL0L12254g (CaALT1)*, *and CAGL0M00550g (CaSTR2)*]. In *C. albicans* cells cultured under biofilm growth conditions only the ortholog of *C. glabrata CAGL0K08580g*, *AAT1*, was found to be positively regulated by Efg1 according to the PathoYeastract (http://pathoyeastract.org/).

2.5. Efg2 controls the expression of genes involved in carbon source and energy metabolism. From the group of genes that was found to be up-regulated by Efg2 in *C. glabrata* cells cultured for 24h under biofilm growth conditions, there is a subset of genes identified as playing a part in carbon and energy metabolism. Using the Kegg mapper tool (http://www.kegg.jp/kegg/mapper.html) five genes were identified as playing a role within the carbon metabolism pathway, namely *CAGL0B03663g (ScCIT2)*, *CAGL0E01705g (CaMDH1)*, *CAGL0H06633g* (which does not have a known ortholog in *C. albicans* or in *S. cerevisiae*), *CAGL0J00451g* (which does not have a known ortholog in *C. albicans* or in *S. cerevisiae*), and *CAGL0L09273g (Sc ICL2)*. The effect of these genes is mainly related to the activation of the glyoxylate cycle. Additionally, some of the genes that are up-regulated by Efg2 seem to up-regulate fatty acid β-oxidation. Simultaneously, Efg2 seems to down-regulate genes involved in the biosynthesis of long-chain fatty acids. Given the up-regulation of genes related to β-oxidation, it was also pertinent to see what the effect of Efg2 in the expression of genes related to peroxisome organization was. The genes *PEX2*, *PEX3*, *PEX4*, *PEX5B*, *PEX7*, *PEX10*, *PEX11*, *PEX12*, *PEX21*, *PEX21B*, *PEX23B*, *PEX24*, *PEX25*, *and PEX32* were all found to be down-regulated in the absence of *EFG2* in *C. glabrata* cells cultured for 24h under biofilm growth conditions. Altogether, these results suggest that *C. glabrata* Efg2 might play a role in cellular adaptation to less nutritious conditions such as the ones experienced by the innermost cells of biofilms, particularly in the use of 2 carbon molecules as an alternative source.

2.6. Efg2 controls the expression of ergosterol metabolism-related genes. Among genes that are up-regulated by Efg2, some were found to be related to the synthesis of ergosterol and other steroids. In *C. albicans* none of the orthologs of these genes were found to be potential targets of Efg1 under biofilm growth conditions. These results seem to suggest that Efg2 up-regulates the expression of genes involved in the biosynthesis of steroids. These results further suggest that Efg2 might be implicated in the resistance to azoles and polyenes, as these drugs effectiveness depend on ergosterol biosynthesis.

2.7. Efg2 controls the expression of cell wall metabolism-related genes. Although the "cell wall organization" functional category was not evidenced in the results presented above, a subgroup of genes appears to be up-regulated by Efg2 in biofilm conditions. This subgroup contains genes coding for β-mannosyltransferases. The β-1,2-mannosyltransferases are enzymes identified in several yeast species, in particular among *C. albicans*, *C. dubliniensis*, *C. glabrata*, and *C. orthopsilosis*. These enzymes are encoded by the BMT gene family and are responsible for the synthesis of yeast β-mannosides.[108] C. albicans exhibits β (1,2)-oligomannosides on its cell surface. Several cell wall glycoconjugates of *C. albicans*, such as mannoproteins, phosphopeptidomannan, and phosholipomannan, are replaced by terminal β-1,2-mannosides. These enzymes play an important role in cell wall composition, and therefore have implications in the adhesion process that has an important relevance in biofilm formation.

Efg2 is not a determinant of antifungal drug resistance in C. glabrata in planktonic growth. As mentioned above, from the RNA-seq collected data Efg2 was found to control the expression of a high number of multidrug transporters in both biofilm and planktonic conditions. In biofilm conditions Efg2 activates the expression of 12 drug resistance genes, as detailed above, while in planktonic growth conditions the basal expression of 13 drug resistance genes was found to be positively controlled by Efg2, including the ABC drug efflux pump encoding genes *CDR1* and *PDH1*. Additionally, Efg2 was found to regulate the expression of ergosterol biosynthesis genes, which is expected to affect azole and polyene drug activity. This posed the question whether the Efg2 putative TF plays a role in drug resistance in *C. glabrata*. To assess if Efg2 is relevant for the resistance of *C. glabrata* to antifungals, several antifungal compounds were tested. For fluconazole, ketoconazole, amphotericin B and caspofungin no significant differences were observed between strains, however differences were observed for flucytosine.

Table 1 - MIC values estimated for Flucytosine towards C. glabrata strains KUE100, Δefg1, and Δefg2. Results are the mean values of 3 independent experiments.

MIC (mg/L)	Flucytosine
KUE100	1
Δefg1	1
Δefg2	0.25

A possible explanation for the increase of resistance to flucytosine displayed by *C. glabrata* cells in this experiment is the existence of several genes that are putatively down-regulated by Efg2 and whose mutations are described as creating hypersensitivity. Genes that fall into this category are *CAGL0M05885g (CaLAS1)*, *CAGL0L12672g (CaNOP4)*, *CAGL0J00957g (CaRLP24)*, *CAGL0J03476g (ScRSA4)*, and *CAGL0H05709g (CaNOG1)*.

Despite the results obtained for azole and echinocandin resistance, suggesting that Efg2 has no role in the resistance to these antifungal drugs, it will be important to assess MIC_{50} levels in biofilm

conditions. This analysis will allow the evaluation of the eventual role of Efg2 in drug resistance in biofilm cells.

Analysis of the role of Efg2 in the amount of ergosterol in cells grown in biofilm and planktonic conditions. From the RNA-seq data it became evident that several genes that are a part of the ergosterol biosynthetic pathway were up-regulated by the Efg2 gene in biofilm conditions. Additionally, the changes in expression of ergosterol biosynthesis genes in biofilm cells, when compared to planktonic cells, did not allow the formulation of a clear hypothesis, as they appeared to be contradictory, in the sense that some of the genes were up-regulated, while others were down-regulated in these conditions. Ergosterol is known to be a constituent of the plasma membrane of fungi and of the ECM that constitutes the biofilm formed by some *Candida* species, such as *C. albicans*. Therefore, we have quantified the amount of ergosterol present in *C. glabrata* KUE100 and Δ*efg2* cells grown under planktonic and biofilm conditions. Results are shown in Figure 4. From these results it is possible to conclude that Efg2 does not appear to affect total ergosterol concentration, independently of the type of growth to which the cells are subjected. However, it is worth noticing that there is a significant difference between the amount of ergosterol found in planktonic and biofilm cells, with the later exhibiting nearly 3-fold lower concentrations of ergosterol.

It has been reported in *C. albicans* that cells that constitute mature biofilm have low levels of membrane sterol when compared to planktonic grown cells, but have higher expression of genes related to ergosterol biosynthesis. This is thought to be a response to the hypoxic conditions to which biofilm cells are subjected, since several steps of ergosterol biosynthesis require oxygen. [37][38][39]

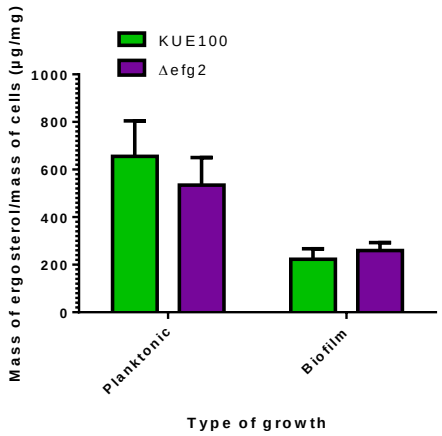

Figure 4 - Average amount of ergosterol per mass of cells of C. glabrata strains KUE100 and Δefg2, after growth under planktonic or biofilm conditions. Results are presented as means and standard deviation of three biological replicates, corresponding to three technical replicates each.

Efg2 affects pseudohyphal differentiation in C. glabrata. Although pseudohyphal differentiation does not seem to take place in *C. glabrata* biofilm cells, *C. albicans* Efg1 was predicted to control this process [40]. Additionally, Efg2 was found to activate the expression of Tec1, which is also predicted to affect this morphological change, based on the role of its *C. albicans* ortholog [12]. Significantly, the Efg2 target genes *SFL2* and *CHP2* have been described as playing a role in the induction of hyphal genes in *C.albicans* cells [41][42].

With the goal of finding out whether the Efg2 gene plays a role in *C. glabrata* differentiation process into pseudohyphae, cells of the KUE100 strain and of the mutant Δ*cgefg2* were grown in YPD medium with isoamyl alcohol to stimulate the differentiation process. After 48 hours of growth, 7 µL of each cell culture were observed under the microscope. Three biological replicates were done, and for each replicate, several images were acquired. A total of 5195 cells from the wt strain and 2626 from the Δ*efg2* strain were analyzed. For each replicate, a percentage of pseudohyphal cells was computed for each strain. Results are presented in Figure 5

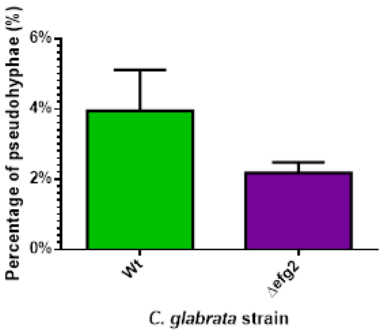

Figure 5 - Average percentage of pseudohyphal formation by C. glabrata wt and Δefg2 and standard deviation.

Even though the difference observed between the degree of formation of pseudohyphae between the two *C. glabrata* strains was not statistically significant it has to be taken into account that only two assays were considered. Therefore, these results should be seen as preliminary and further assays should be performed to corroborate these results. Nevertheless, there appears to be a 2-fold decrease in the degree of pseudohyphal formation in the absence of *EFG2*, suggesting that Efg2 might play a role in this differentiation process.

Efg2 subcellular localization in C. glabrata. Efg2 is predicted to be a transcription factor since its *C. albicans* ortholog Efg1 is known to be one. Transcription factors are synthetized in the cytoplasm and have to be imported to the nucleus in order to perform their function. This process is known to be modulated by several mechanisms such as direct phosphorylation, which masks the nuclear localization signals required for the transport of transcription factors to the nucleus, binding to an anchoring protein, which keeps the transcription factor retained in the cytoplasm, modulation of the import machinery, and very likely a conjugation of more than one of these mechanisms.[43] With the goal of finding whether the Efg2 gene was localized to the nucleus its subcellular localization was assessed using the GFP fusion that was created by the use of the pGreg576 in L5U1 cells. In this localization assay L5U1 cells transformed with pGreg576 plasmid (L5U1+VV) were compared with L5U1 cells transformed with the pGreg576 that was cloned with the MT-I promoter sequence and the Efg2 gene (L5U1+pGreg576+MT-I+Efg2).

Since the main goal of this work is to understand the role of Efg2 gene in the process of biofilm formation, cells grown in planktonic conditions were compared to cells that were grown in biofilm for 6h and for 24h. For all the tested conditions no specific subcellular location was observed, giving no further clues to the mechanisms of activation of this gene.

In silico prediction of Efg2 binding sites. To predict the nucleotide sequence recognized and bound to by Efg2 the up-stream regions of its up-regulated genes (in planktonic and biofilm growth), were obtained using the PathoYeastract (http://pathoyeastract.org/). Consensus motifs were predicted from those up-stream regions using DREME (Discriminative Regular Expression Motif Elicitation) online software (http://meme-suite.org/doc/dreme.html?man_type=web). A total of twenty-nine or fifteen motifs were obtained considering the Efg2 activated genes in biofilm cells or in planktonic cells, respectively. Since this still was a very high number of motifs to further test, a screen-down process was necessary.

First the TATA boxes were eliminated since these are transcription initiation sites present in all promoter regions. After this first filtering process several factors were considered simultaneously to help select the most relevant predicted motifs. The chosen factors were presence in an higher number of up-stream regions of genes, presence in groups of genes described as biofilm-related, presence in genes up-regulated by Efg2 in planktonic and biofilm conditions simultaneously, and similarity to the binding sites described for Efg1 in *C. albicans*. After the filtering process three motifs were chosen. These motifs are presented in Table 2.

Table 2 - Predicted binding sites for C. glabrata Efg2 TF.

Predicted binding motifs for *C. glabrata* Efg2
CGATGS
CCATTGTY
CASAGAA

This prediction still requires experimental testing.

Conclusion

In this work the transcription facto Efg2, but not its close homolog Efg1, was found to play a role in the adhesion of *C. glabrata* to both biotic and abiotic surfaces, leading to biofilm formation.

Changes in gene expression suffered by *C. glabrata* cells when cultured under biofilm growth conditions were studied through RNA-seq analysis. The complexity of the process of biofilm formation by *C. glabrata* is evidenced by the large number of genes differently expressed (3072), which accounts for approximately half of the *C. glabrata* genome. Among the differently expressed genes, more than 17% belong to the category "unknown". This results partly from the lack of homology of *C. glabrata* and *C. albicans*, and *C. glabrata* and *S. cerevisiae*, and from a gap of knowledge on gene function, especially in *C. glabrata*. Future work on *C. glabrata* functional genomics will certainly contribute to a better knowledge of molecular mechanisms underlying biofilm formation by *C. glabrata*.

Among the differentially expressed functional groups in biofilm cells, those specifically related to biofilm formation by *C. glabrata* were further analyzed. From the "stress response" group, the up-regulated genes were found to be involved in heat shock and oxidative stress response, including several encoding transcription factors. These transcription factors are involved in general stress response (*MSN1 and MSN4)*, in oxidative and metal stress response (*AP1, CAD1, YAP6, and YAP7*), in acid/alkaline stress response (*RIM101, HAA1,WAR1*), in unfolded protein response (*HAC1*),and in osmotic stress response (*HOT1*). These results suggest that biofilm *C. glabrata* cells are experiencing adverse conditions and/or preparing to cope with them. Biofilms have been associated to a higher drug resistance when compared to planktonic grown cells. Our RNA-seq analysis supports this observation, since several MDR transporters (e.g.: Cdr1, Qdr2, CgAqr1, CgTpo1_2, CgTpo3) were up-regulated under biofilm growth conditions. Nevertheless, other factors most certainly contribute to the increased resistance displayed by biofilms, such as the presence of an EPS layer surrounding the cells, and the existence of persister cells. *C. glabrata* cells grown under biofilm conditions also display an up-regulation of several adhesins. This up-regulation supports the notion that adhesion is required during biofilm growth for cell-to-cell and cell-to-surface attachment, making the biofilm more robust and resistant to environmental stressors. In *C. albicans*, transcription factors Efg1 and Tec1 are known to play an important role in biofilm formation. The *C. glabrata* orthologs, encoded by ORFs *CAGL0M07634g* and *CAGL0M01716g* respectively, were both up-regulated under biofilm growth conditions.

The formation of biofilms by *C. glabrata* is associated with changes in the physiology of the organism. This is evidenced by our results showing the up-regulation of genes of the YPS family involved in cell-wall remodeling as an evasion mechanism from the immune system of the host. Our results also indicate changes in amino acid metabolism towards amino acid deamination, suggesting that biofilm cells are subject to nitrogen starvation. The carbon metabolism also appears to be shifted towards the usage of 2-carbon molecule sources, as indicated by the up-regulation of genes of the glyoxylate cycle and down-regulation of glycolysis. This hypothesis is strongly supported by the up-regulation of genes involved in β-oxidation, together with the up-regulation of genes involved in the assembly of peroxisomes. No clear picture could be drawn from the changes in the levels of expression of genes involved in ergosterol biosynthesis, although our results suggest that this pathway is skewed towards the formation of intermediates. The biosynthesis of (GPI)-anchors, involved in adhesion, were found to be down-regulated, possibly due to the specific stage of development of the biofilm studied. Figure 6 summarizes the main metabolic changes occurring in *C. glabrata* cells growing as biofilm

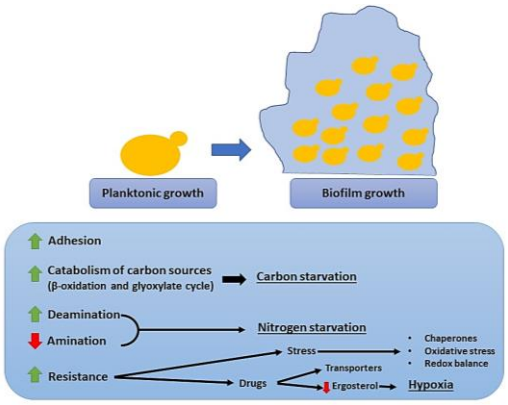

Figure 6 -Summary of the main metabolic changes occurring in C. glabrata cells when cultured in biofilm conditions as revealed by RNA-seq analysis.

The role of Efg2 in biofilm formation in *C. glabrata* was studied by RNA-seq analysis. This study revealed Efg2 as putatively responsible for the control of approximately 1/3 of the genes up-regulated under biofilm conditions. This overlap highlights *EFG2* as a main regulator of the adaptation of *C. glabrata* cells to biofilm growth conditions. Efg2 appears to up-regulate genes encoding adhesins, which is likely one of the main reasons why Δ*efg2* cells display decreased adhesion to epithelial cells and decreased biofilm formation. Efg2 was also found to be upregulation of drug resistance and stress response genes, as well as in the synthesis of ergosterol.Interestingly, biofilm cells were found to contain decreased levels of ergosterol, a likely consequence of hypoxia felt by the *C. glabrata* cells in the inner parts of the biofilm. This physiological change was, however, shown to happen independently from Efg2. Drug susceptibility assays under planktonic conditions suggest that Efg2 only plays a role in resistance to flucytosine, but not other antifungal drugs. Further susceptibility assays should however be performed to unveil the role of Efg2 in the resistance to antifungal drugs in *C. glabrata* cells grown under biofilm conditions.

Efg2 was further found to affect pseudohyphal differentiation. However, since only two assays were performed these results should be considered as preliminary, and further testing is required to reach statistical significance. Subcellular localization did not prove to be helpful in the understanding of Efg2 action, given that Efg2 was found to be localized throughout the whole cell. An *in silico*

prediction of the binding site of Efg2 was performed and three motifs were considered as more probable, “CGATGS”, “CCATTGTY”, and “CASAGAA”. This prediction still requires experimental confirmation. Altogether, our results strongly suggest that *C. glabrata* Efg2 is a TF playing a key role in biofilm formation by this yeast. A model showing the hypothesized role of Efg2 as a TF binding to the motives predicted in this work and regulating genes involved in biofilm formation is presented in Figure 7.

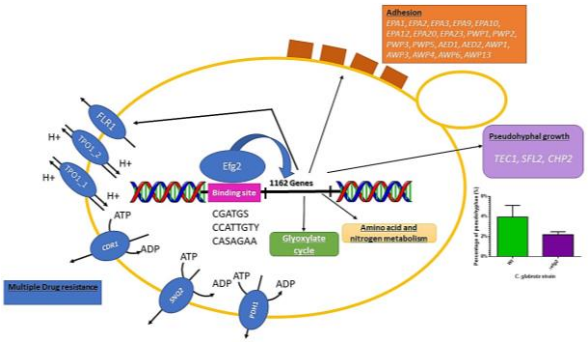

Figure 7 -Hypothetical model of action of Efg2 in C. glabrata cells cultured under biofilm conditions.

Among the 1162 genes found to be regulated by Efg2, several encode for putative transcription factors. Figure 27 summarizes the TF grouped in functional categories considered relevant for biofilm formation and suggesting that Efg2 is a key player in a very complex transcription regulatory network controlling this multifactorial phenomenon. This work contributed to further understand the mechanism underlying *C. glabrata* adaptation to biofilm growth conditions and to unveil the role of Efg2 in the complex process that is biofilm formation by *C. glabrata*. It is also a step forward in the development of new preventative methods since Efg2 is a promising drug target to prevent biofilm formation.

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