Biofilm formation in *Candida glabrata:* the role of the Transcription Factor Tec1

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C. glabrata is the second most prevalent cause of human candidiasis. One of the factors underlying the colonization and infection by this pathogen is its ability to form resilient biofilms. It is, thus, important to understand the molecular basis behind this phenomenon, to guide the design of more successful therapeutic options. This work aimed to understand the role and the mechanisms of action of Tec1 (ORFCAGL0M01716g) in biofilm formation. Phenotypic analysis showed that Tec1 is intimately involved in biofilm formation and adhesion to biotic surface. The transcriptomic remodeling occurring in cells upon 24 h of biofilm formation, and the role of Tec1 in these changes, were analysed through RNA-sequencing. Tec1 was found to control one third of the differentially expressed genes in biofilm, including adhesion, cell wall organization, ergosterol biosynthesis, carbon and nitrogen metabolism and stress and drug resistance. Tec1 was found to control the step-wise activation of the adhesins *CgAED2*, *CgPWP5* and *CgAWP13*, occurring upon 6 h, 24 h and 48 h of biofilm formation, suggesting a particular importance in later stages of biofilm formation. Furthermore, Tec1 was shown to regulate the decrease in ergosterol content registered in biofilm cells, when compared to those growing in planktonic cultivation. Among the targets of Tec1, *CgAUR1* gene was found to play a role in biofilm formation. Finally, in silico analysis revealed the possible Tec1 recognition sequences "CAATGGBA", "CAMATACA", "CGATGSCC" and "GCGATGAS". These results highlight the important role played by Tec1 in C. glabrata biofilm formation, making it a promising new target for antifungal therapeutics.

Keywords: *Candida glabrata*, *TEC1*, biofilm formation, RNA-seq

INTRODUCTION

Candida glabrata is a commensal colonist of the intestinal tract in humans, being an opportunistic pathogen.[1] Epidemiological studies have shown that more than 250,000 people worldwide are affected by invasive candidiasis (IC) annually, resulting in more than 50,000 deaths.[2] *C. glabrata* is the second most common cause of this kind of infections and immunocompromised patients are the major group of risk.[3,4] As virulence factors, *C. glabrata* displays the ability to survive within macrophages, to synthesize adhesins and secrete phospholipases. In fact, *C. glabrata* exhibits the unusual capacity to multiply within phagocytic cells, such as neutrophils or macrophages, and is reported to use the macrophages as "Trojan‐Horses" in order to infiltrate the host.[4] Additionally, it is a high stress tolerant and very robust species, since it can not only survive on host tissues but also on inanimate surfaces for more than 5 months and this is due to its capability to form multilayered biofilms.[5,6] Cases of failure to eradicate *C. glabrata* infections are expanding due to resistance to antifungal drugs, which is alarming considering the limited number of drug classes that target different fungal components.[7]

Biofilm formation ability contributes to *C. glabrata* persistence and results in a low therapeutic response and serious recurrent candidiasis. For their complex organization, biofilms are very resistant to antifungal treatments.[8] Thus, new approaches to the treatment of *C. glabrata*'s biofilms are needed and, in line with this, there is increasing interest in understanding the transcriptional network responsible for this phenomenon.

Nobile *et al.* (2012)[9] identified important transcription factors (TFs) essential for biofilm formation using a *Candida albicans* library of 165 TF deletion mutants that were tested for biofilm growth *in-vitro* and *in-vivo* on rat denture and catheter models. The identified TFs were Tec1, Efg1, Bcr1, Brg1, Rob1 and Ndt80. Of the target genes regulated by these TFs, almost half were controlled directly by two or more regulators. This remarkable study has shown that multiple transcriptional regulators regulate the expression of key biofilm effector genes. These TFs not only regulate their own expression but also the expression of the remaining members of the network.[9,10] Even though there is a high amount of information available regarding the mechanisms that control biofilm formation in *C. albicans*, much scarce information is available for *C. glabrata*.

The *C. albicans* Tec1 was shown to be a positive regulator of morphogenesis, required for hyphal formation and also an important regulator of biofilm formation, recently considered a member of the transcriptional enhancer activator (TEA/ATTS) family of TFs. *C. albicans Δtec1* null mutant was shown to form much less biofilm compared with the wild type strain, while the type of cells composing the biofilm were exclusively yeast cells, contrasting with the hyphal filaments on the wild type biofilm.[11] Contrasting with the knowledge on the biological roles played by *C. albicans* Tec1 TF, in *C. glabrata* the biological functions of its predicted orthologs Tec1 (ORF *CAGL0M01716g*) and Tec2 (ORF *CAGL0F04081*) remain unclear.

Our research group enrolled a study to find if these TFs were also related to biofilm formation in *C. glabrata*. Preliminary unpublished results suggest that Tec1 (ORF *CAGL0M01716g*) is also related to biofilm formation in *C. glabrata*, but the same didn't happen for Tec2 (ORF *CAGL0F04081*).

Motivated by this hypothesis, this dissertation was outlined in order to clarify and improve the knowledge about the role and the mechanisms of action of the *C. glabrata* Tec1 TF in its ability to form stable biofilms and to unveil possible targets for the future development of strategies to prevent biofilm-based pathogenicity by *C. glabrata*.

MATERIALS AND METHODS

Strains, plasmids and growth media. The parental strain *C. glabrata* KUE100 and derived single deletion mutants: *Δtec1, Δpup1, Δaur1, Δaed2, Δpwp5, Δbmt1, Δbmt7 and Δawp13* were kindly provided by Prof. Hiroji Chibana, Medical Mycology Research Center, Chiba University, Chiba, Japan. *C. glabrata* L5U1 strain (*Δcgura3Δcgleu2*) was kindly provided by John Bennett[12] of the National Institute of Allergy and Infectious Diseases, NIH, Bethesda, MD. Additionally, the CBS138 *C. glabrata* strain, whose genome sequence was released in 2004, was used in this study for gene amplification purposes. The

plasmid pGREG576 was obtained from the Drag & Drop collection.[13]

The VK2/E6E7 human epithelium cell line ATCC® CRL-2616™, used for adhesion assays, is derived from the vaginal mucosa of a healthy premenopausal female submitted to vaginal repair surgery. *C. glabrata* cells were batch-cultured at 30ºC with orbital agitation (250 rpm) in different growth media according to the following protocols: The yeast extract peptone dextrose (YPD) growth media was used as a rich medium, with the following composition (per liter): 20 g glucose (Merck), 10 g yeast extract (HIMEDIA) and 20 g bacterial-peptone (Dickson). The minimal growth medium (MMG) used contained per liter: 20 g glucose (Merck); 2.7 g $(NH_4)_2SO_4$ (Merck); 1.7 g yeast nitrogen base without amino acids or $(NH_4)_2SO_4$ (Difco). Also used was Sabouraud's dextrose broth (SDB) pH 5.6 containing 40 g glucose (Merck) and 10 g peptone (LioChem) per liter. Roswell Park Memorial Institute (RPMI) 1640 growth medium pH 4 contained per 600 mL: 6.24 g RPMI 1640 (Sigma); 20.72 g 3-(N-morpholino) propanesulfonic acid (MOPS) (Sigma); 10.8 g glucose (Merck).

Quantification of biofilm formation. In order to assess the capacity of biofilm formation of *C. glabrata* cells, the Presto Blue assay was used. Cells were grown in SDB medium or MMG-U medium (composed of minimal medium supplemented with 60 mg/L Leucine) and collected at mid-exponential phase. A cell suspension was prepared with an OD_{600nm} of 0.1. Cells were then inoculated in 96-well polystyrene titter plates (Greiner), which were previously filled with the appropriated medium, SDB at pH 5.6 or RPMI at pH 4, in order to have an initial OD_{600nm} = 0.05±0.005. Afterwards, cells were sealed with a membrane (Greiner Bio-One) and cultivated at mild orbital shaking (70 rpm), for 24h, at 30ºC. Subsequently, each well was washed two times with 100 µL of sterile PBS pH 7.4 [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)] to remove the cells that were not attached to the formed biofilm. After washing, Presto Blue reagent was prepared in a 1:10 solution in the medium used for biofilm formation, adding 100 µL of the solution to each well. Plates were incubated at 37ºC for 30 min. Afterwards, absorbance reading was determined in a microplate reader (SPECTROstar Nano, BMG Labtech) at the wavelength of 570 nm and 600 nm for reference.

Adhesion to human vaginal epithelium cells. For the epithelium adhesion assays, VK2/E6E7 human epithelium cells were grown adhered to an abiotic surface and further detached in order to be inoculated in 24-well polystyrene plates (Greiner) with a density of $2.5x10^5$ cell/mL. Additionally, *Candida glabrata* cells were inoculated with an initial OD_{600nm} = 0.05±0.005, cultivated at 30 ºC, during 16±0.5 h, with orbital shaking (250 rpm). In order to initiate the assay, the culture medium of mammalian cells was removed and substituted by new culture medium, in each well, and, subsequently, *C. glabrata* cells were added to each well, with a density of $12.5x10^5$ CFU/well. The plate was centrifuged for 1 min at 1000 rpm and room temperature. Then, cells were incubated at 37°C, 5% CO_2, for 30 min. Afterwards, each well was washed 3 times with 500 µL of PBS pH 7.4, following the addition of 500 µL of Triton X-100 0.5% (v/v) and incubation at room temperature for 15 min. The cell suspension in each well was then recovered and spread onto agar plates to determine Colony Forming Units (CFU) count, which represent the proportion of adherent cells to the human epithelium.

C. glabrata transformation. For transformation purposes, *C. glabrata* L5U1 cells were batch-cultured at 30°C, with orbital agitation (250 rpm) in liquid rich medium YEPD until a standard OD_{600nm} 0.4 ± 0.04 was reached. All transformation reactions were performed using the Alkali-Cation Yeast Transformation Kit (MP Biomedicals), according to the manufacturer's instructions. Cells were then transferred to microcentrifuge tubes, combining: 100 µL yeast cells, 5 µL Carrier DNA, 5 µL Histamine Solution and 100-200 ng plasmid DNA. Cells were gently mixed and incubated at room temperature for 15 min. A mixture of 0.8 mL PEG and 0.2 mL TE/Cation MIXX solution was added to each transformation reaction, followed by 10 min incubation at 30 ºC and heat shock at 42ºC for 10 min. Cells were then pelleted in a microcentrifuge and resuspended in 100 µL YEPD liquid medium before plating in appropriate medium agar plates.

RNA-sequencing analysis. Cells for RNA-seq analysis were grown in SDB medium. Planktonic cells were cultured at 30ºC with orbital agitation (250 rpm), while biofilm cells were cultured at 30ºC, in square Petri dishes, with orbital agitation (70 rpm). Three independent total RNA isolates were extracted from wild type and single deletion mutant cells during planktonic exponential growth and upon 24h of biofilm growth. Total RNA was isolated using an Ambion Ribopure-Yeast RNA kit, according to manufacturer's instructions. Cell cultures and RNA extraction were performed by Mafalda Cavalheiro. 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. Prior to RNA-seq analysis quality control measures was implemented. Concentration of RNA was ascertained via fluorometric analysis on a Thermo Fisher Qubit fluorometer. Overall quality of RNA was verified using an Agilent Tapestation instrument. Following initial QC steps sequencing libraries were generated using the Illumina Truseq Stranded Total RNA library prep kit with ribosomal depletion via RiboZero Gold according to the manufacturer's protocol. Briefly, ribosomal RNA was depleted via pull down with bead-bound ribosomal-RNA complementary oligomers. The RNA molecules were then chemically fragmented and the first strand of (cDNA) was generated using random primers. Following RNase digestion, the second strand of cDNA was generated replacing dTTP in the reaction mix with dUTP. Double stranded cDNA then underwent adenylation of 3' ends following ligation of Illumina-specific adapter sequences. Subsequent PCR enrichment of ligated products further selected for those strands not incorporating dUTP, leading to strand-specific sequencing libraries. Final libraries for each sample were assayed on the Agilent Tapestation for appropriate size and quantity. These libraries were then pooled in equimolar amounts as ascertained via fluorometric analyses. Final pools were absolutely quantified using qPCR on a Roche LightCycler 480 instrument with Kapa Biosystems Illumina Library Quantification reagents. Sequencing was performed on an Illumina HiSeq 3000, producing 2x150 bp paired-end reads, 2 gigabases (GB) clean data, yielding 52 million reads per sample. Paired-end reads were obtained from wild type (*C. glabrata* KUE100) and correspondent deletion mutant strain (*CAGL0M01716g*). Two replicates of each sample were obtained from three independent RNA isolations, subsequently pooled together. Samples reads were trimmed using Skewer[14] and aligned to the *C. glabrata* CBS138 reference genome, obtained from the *Candida* Genome Database (CGD)[15], using TopHat. HTSeq[16] was used to count mapped reads per gene. Differentially expressed genes were identified using DESeq2[17] 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. Significantly differentially expressed genes were clustered using hierarchical clustering in R[18]. *Candida albicans* and *Saccharomyces cerevisiae* homologs were obtained from the CGD and Saccharomyces Genome Database (SGD)[19], respectively. Raw data pre-treatment was performed by Pedro Pais, in collaboration with Geraldine Butler, University College Dublin.

Transcriptomic data analysis. The RNA-sequencing analysis provided two datasets: wild type vs *Δtec1* deletion mutant in planktonic growth and wild type planktonic vs wild type biofilm growth. The genes of each dataset were submitted to several analysis using different databases and bioinformatic tools, so they could be grouped according to their biological functions. This was accomplished mainly by resorting to the description of the C. glabrata genes found on the CGD (http://www.candidagenome.org/). The uncharacterized genes were clustered based on the description of ortholog genes in S. cerevisiae or in *C. albicans*, according to the SGD or in CGD, respectively. Go-Stats from GoToolBox web server[20] allowed the determination of the main Gene Ontology (GO) terms to which the genes were related. The bioinformatic tool Kyoto Encyclopedia of Genes and Genomes (KEGG) Mapper[21] was used to analyze the main metabolic pathways to which the genes were related to. The KEGG organism code chosen was "cgr" for *C. glabrata*. The functional protein association networks tool, STRING[22], helped to understand the protein-protein interactions and its cellular functions. From this organization, several genes related to cell adhesion were chosen for the following gene expression analysis. For the analysis of the possible Tec1 recognition sequences, a search for consensus sequences in the upstream regions of each positively regulated gene in biofilm was performed. By resorting to PathoYeastract[23] database, these upstream regions were obtained and then, these were used in DREME (Discriminative Regular Expression Motif Elicitation)[24] informatic tool. I-TASSER (Iterative Threading ASSEmbly Refinement)[25] approach was used for Tec1 structure and ligand binding residues prediction. The InterPro[26] database was used to search for conserved domains in Tec1 and Clustal Omega[27] program (https://www.ebi.ac.uk/Tools/msa/clustalo/) allowed the multiple alignment of the three *TEC1* orthologs from C. albicans, S. ceresiviae and *C. glabrata*.

Gene expression analysis. The quantitative Real Time Polymerase Chain Reaction (RT-qPCR) technique was used in order to estimate the expression levels of *CgPWP5*, *CgAED2* and *CgAWP13* genes encoding adhesins, by visualizing the abundance of mRNA transcripts in each sample. The total RNA extraction was performed for *C. glabrata* KUE100 and *Δtec1* single deletion mutant strains cells grown in planktonic and biofilm growth conditions, according to the Hot-phenol method described by Kohrer & Domdey[28]. For the first step of this method, the pellets were resuspended in 900 µL of AE buffer (50 mM NaAc (Sigma), 10mM EDTA (Aldritch), pH=5.3; 0.1% (v/v) diethylpyrocarbonate (DEPC) treated). Afterwards, it was added 90 µL of SDS 10% (w/v) (Sigma) and 800 µL of phenol, following a short vortex of 5 sec. Then, samples were incubated at 65 ºC for 4 min and transferred into dry ice until the formation of crystals was identified. The mixtures were centrifuged at 15000 rpm, at 4 ºC for 5 min. The upper phase was collected to new microcentrifuge tubes. Subsequently, a two-step extraction with phenol was performed where for each step 400 µL of a 25:24:1 phenol/chloroform/isoamilic acid solution (Sigma) was added. A short vortex was carried out, following a centrifugation at 15000 rpm, at 4 ºC for 5 min. The top phase was collected to new microcentrifuge tubes. Then, a final extraction was performed, using 800 µL of 24:1 chloroform/isoamilic acid solution (Sigma), following vortex and centrifugation in the same conditions, finishing with the collection of the top phase. After this stage, a 1/10 of the final volume of the mixture obtained by centrifugation of sodium acetate 3 M (Merck, pH=5.3, 0.1% DEPC treated) was added. Then a purification step was performed by adding 1 mL of cold ethanol 100%. After a short vortex, the samples were stored in -20˚C for 20 min. Then, a prolonged centrifugation was carried out at 15000 rpm, at 4ºC for 20 min. The liquid phase was discarded and the remaining precipitates were washed with 750 µL of cold ethanol 70% (v/v) and centrifuged at 15000 rpm, at 4 ºC for 20 min. The liquid phase was carefully discarded, and afterwards, in order to preserve the formed precipitates, a drying step at Speed Vacuum Concentrator Plus (Eppendorf) was performed for 15 min. Then, the material was resuspended in 50 µL of sterile deionized water 0.1% (v/v) DEPC treated and the volume was divided by two aliquots of 10 µL and 40 µL. The aliquots of 10 µL were used to assess the purity and quantify total RNA concentration in a NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies). At last, the samples were diluted in order to have a concentration of 500 ng/µL for the real time RT-PCR. The SYBR® Green fluorescence is detected by the 7500 Real-Time PCR Systems (Applied Biosystems®), following the registration performed by the software 7500 Systems SDS Software from Applied Biosystems in the amplification plot. *CgACT1* was used as housekeeping gene.

Quantification of total cellular ergosterol. Total ergosterol content was extracted from *C. glabrata* KUE100 and *Δtec1* single deletion mutant cells using the method of physical disruption[29] with some adjustments. Planktonic growth condition cells were cultivated in 100 mL of YEPD and with an orbital agitation of 250 rpm until stationary phase was reached. Biofilm growth condition cells were grown in SDB medium and collected at mid-exponential phase. Cells were then inoculated in square polystyrene titter plates filled with 40 mL of SDB medium at pH 5.6, to have an initial OD_{600nm} = 0,05±0.005. Afterwards, cells were cultivated at mild orbital shaking (70 rpm), for 24h, at 30ºC. The cultures were conducted in triplicates. Planktonic cells were harvested by centrifugation and ressuspended in 5 mL of methanol and biofilm cells were harvested by discarding the medium, leaving only biofilm cells in the plates, then 3 mL of sterile water were added and the plate was scraped. The resultant suspension of cells was transferred to a centrifuge tube. This process was repeated and the suspension was centrifuged and and ressuspended in 5 mL of methanol. The Cholesterol, used as an internal standard to allow quantification of the yield of ergosterol extraction, was added in order to have a final concentration of 1 mg/mL in each sample. Afterwards, glass beads were added approximately in the same weight as the cell pellet. Then, each sample was homogenized in 30 sec, following an orbital agitation of 320 rpm for 1 h. The samples were centrifuged at 8000 rpm for 7 min at 4ºC. 1.7 mL of supernatant was extracted to a microcentrifuge tube, following another centrifugation at 11000 rpm for 10 min at 4ºC. 1 mL of the supernatant was then collected and stored until analysis. The extracts obtained were analyzed by High Pressure Liquid Chromatography with a 250 mm x 4 mm C18 column (LiChroCART Purospher STAR RP-18 end- capped 5 mm) at 30ºC. The samples were eluted in 100% methanol at a flow rate of 1 mL methanol per min. Cholesterol was detected at 210 nm corresponding to a retention time of 14.08±0.21 min. Ergosterol was detected at 282 nm with a retention time of 11.71±0.17 min. The corresponding results are presented as the ratio between the average concentration of ergosterol of the *C. glabrata* KUE100 strain or *Δtec1*, according to each case, and the concentration of the other samples tested.

Statistical analysis. Statistical analysis of all data was performed using Graphpad Prism Software version 6.0 (La Jolla, CA, USA). *P*-values were calculated performing one-way ANOVA tests on Microsoft® EXEL 2016. *P*-values equal or inferior to 0,05 were considered statistically significant.

RESULTS

Tec1 is a determinant of biofilm formation in polystyrene surface

To consolidate the previous observations, quantification of biofilm formation in polystyrene surface upon the deletion mutant

Δtec1 was performed using the PrestoBlue cell viability assay, in both SDB and RPMI media. The deletion of this TF in *C. glabrata* was found to reduce biofilm formation in 30% or 10%, in cells cultivated in SDB or RPMI media, respectively (**Figure 1**).

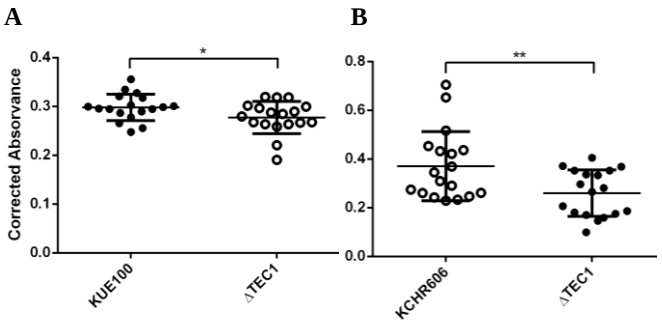


Figure 1- Biofilm formation followed by Presto Blue Cell Viability Assay and measurements of absorbance at 570 nm and 600 nm for reference for the *C. glabrata* KUE100 and *Δtec1* strains. Cells were grown for 24 h and the experiment was performed in RPMI medium (A) and SDB medium pH 5.6 (B). In the scatter dot plot represented each dot corresponds to the level of biofilm formed in each sample. The indicated values are averages of at least three independent experiments. Error bars represent the corresponding standard deviations. ** *P*<0,01; * *P*<0,05.

To further assess if the over-expression of the *CgTEC1* gene would lead to an increase in biofilm formation on polystyrene surface, the quantification of biofilm formation was performed using the L5U1+vv (*C. glabrata* L5U1 cells transformed with the pGREG576 plasmid) and L5U1+*CgTEC1* (*C. glabrata* L5U1 cells transformed with the pGREG576 plasmid containing *PDC1* promotor and *CgTEC1* gene) strains, through the Presto Blue assay. The overexpression of *CgTEC1* resulted in a significant increase in biofilm formation in both media, 73% in SDB and 28% in RPMI, when compared to the parental strain, harboring the empty vector pGREG576 (**Figure 2**).

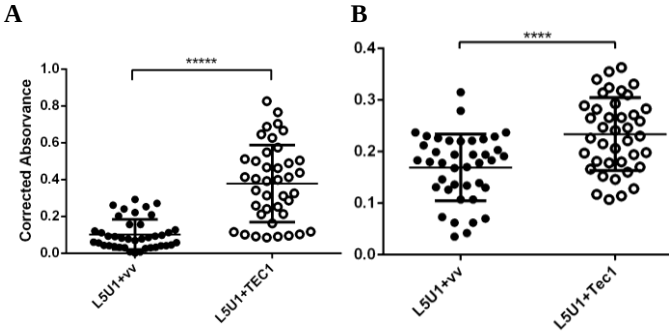


Figure 2- Biofilm formation followed by Presto Blue Cell Viability Assay and measurements of absorbance at 570 nm and 600 nm for reference for the *C. glabrata* L5U1+vv and L5U1+*CgTEC1* strains. Cells were grown for 24 h and the experiment was performed in RPMI medium (A) SDB medium pH 5.6 (B). In the scatter dot plot represented each dot corresponds to the level of biofilm formed in each sample. The indicated values are averages of at least three independent experiments. Error bars represent the corresponding standard deviations. ***** *P*<0,00001; **** *P*<0,0001.

Both experiments reinforce the notion that Tec1 is a key player in *C. glabrata* biofilm formation.

Tec1 is required for C. glabrata adhesion to human vaginal epithelium cells

As an attempt to understand the impact of Tec1 on the capacity of *C. glabrata* cells to adhere to the human vaginal epithelium cells, *C. glabrata* KUE100 and *Δtec1* deletion mutant strains were allowed to contact to VK2/E6E7 human epithelium cells previously inoculated in 24-well polystyrene plates for 30 min at 37°C and 5% CO_2. The percentage of adhesion was then calculated for each replicate, representing the percentage of adhered *C. glabrata* cells, by the ratio between the CFU/ml recovered after incubation with the epithelial cells and the initial CFU/ml for each suspension (**Figure 3**).

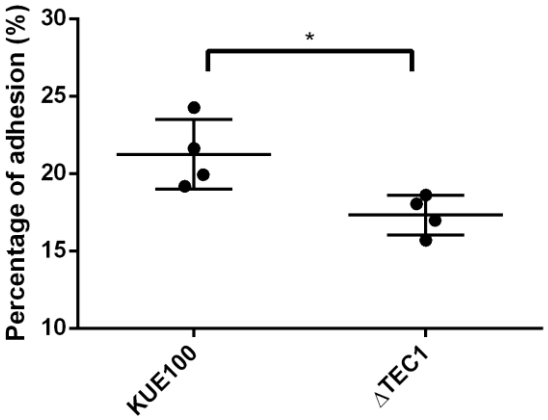


Figure 3- Adhesion capacity to the human vaginal epithelium cells for the KUE100 and Δ*tec1* strains. Cells were cultivated for 16±0.5 h in YEPD medium. *C. glabrata* cells were added to the human cells and incubated at 37°C, 5 % CO_2, for 30 min. The cell suspension in each well was then recovered and spread onto agar plates to determine CFU count. In the scatter dot plot represented each dot corresponds to the proportion of adherent cells to the human epithelium. The indicated values are averages of at least three independent experiments. Error bars represent the corresponding standard deviations. *P<0.05.

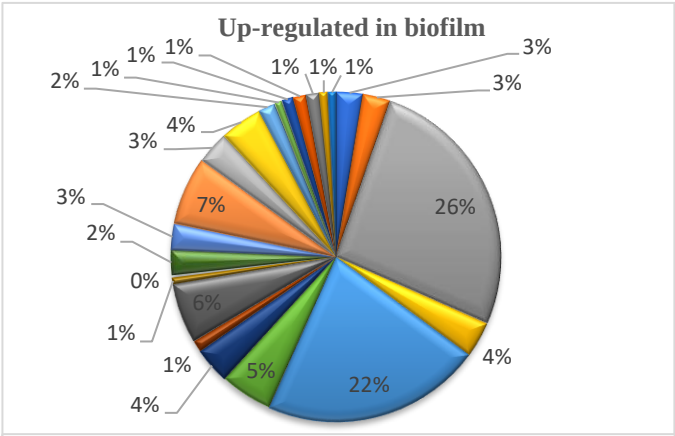
The deletion of *CgTEC1* was found to lead to a 18% reduction in the biofilm formed on top of the epithelial cell layer. This highlights the importance of the TF Tec1 in regulating cell adhesion, even to biotic surfaces.

Transcriptomics analysis of the role of Tec1 in biofilm formation

The effect of *Δtec1* deletion in the transcriptome-wide response of *C. glabrata* cells to biofilm growth was assessed through RNA-sequencing. The RNA-seq analysis started with the global gene expression changes observed in cells cultivated as biofilm for 24 h, when compared to the gene expression profile of planktonic cultivation, giving a dataset with roughly 3000 differentially expressed genes. These genes were further grouped by biological function so we could uncover the most important processes underlying biofilm formation (**Figure 4 A**). The great amount of genes and the diversity of biological processes happening in the cell gives us the idea that biofilm formation is a very complex process. Some functional groups were then selected and more in dept analyzed, considering their expected importance for biofilm formation. In the group named adhesion and biofilm formation, some genes encoding adhesins were observed to be activated, namely the well known Epa family, but also the Pwp, Aed and Awp families of adhesins, all already reported as important for adhesion and biofilm formation in *C. glabrata.* In this group there can be also noted the presence of the *CgTEC1* gene, as well as the *CgEFG1*. Additionally, the response to stress was also shown to be important, with activation of genes involved in heat-shock, oxidative stress response and redox balance, as well as a number of other TFs that act on general stress response, unfolded protein response, acid/alcaline stress among other. Interestingly there is also up-regulation of multidrug-responsive genes, as *CgQDR2* and *CgTPO1_2* drug transporters shown by our group to be important in biofilm formation. Nevertheless, the increase in expression of these multidrug transporters in biofilm with no drug exposure is still to be disclosed. Furthermore, there was also up-regulation of genes related to ergosterol biosynthesis.

Concerning the changes in amino acid and nitrogen metabolism group, it can be observed a tendency to the activation of some deamination steps and inhibition of amination steps, leading to an accumulation of the amino acid precursors α-oxoacids, which is

A



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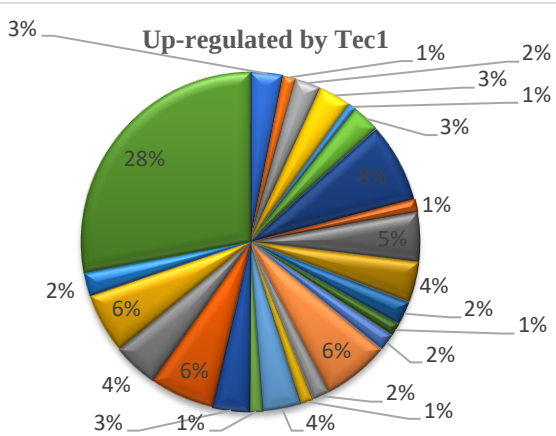

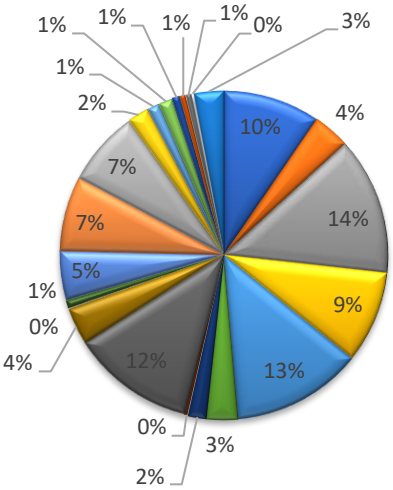

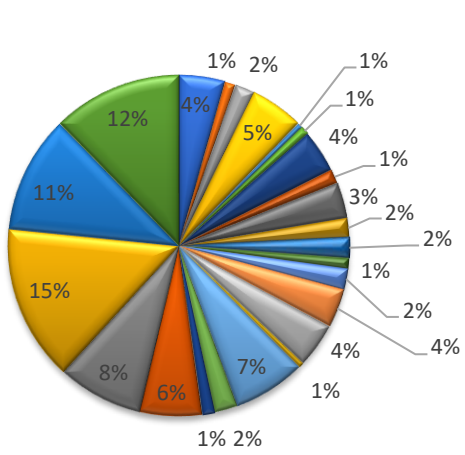

- Intracellular trafficking
- Cell wall organization
- RNA metabolism and translation
- DNA metabolism and repair
- Unknown function
- Response to stress
- Cellular ion homeostasis
- Response to drugs
- Cell cycle
- Cytoskeleton organization
- Virulence
- Adhesion and biofilm formation
- Lipid metabolism
- Carbon and energy metabolism
- Protein metabolism
- Aminoacid metabolism
- Nucletide metabolism
- Cofactor and vitamin metabolism
- Ergosterol biosynthesis
- Nitrogen metabolism
- Autophagy
- Peroxisome biogenesis and organization
- Mitochondrion organization

- Intracellular traficking
- Endocytosis
- Organelle organization
- Lipid metabolism
- Ergosterol biosynthesis
- Response to drugs
- Response to stress
- Cytoskeleton organization
- Cell wall organization
- Adhesion and biofilm formation
- Invasive filamentous growth and virulence
- Co-factor and vitamin biosynthesis
- Cellular ion homeostasis
- Carbon and energy metabolism
- Mitochondrion organization
- Nitrogen metabolism
- Aminoacid metabolism
- Purine and pyrimidine metabolism
- Autophagy
- Protein metabolism
- RNA metabolism and export
- Cell cycle
- DNA metabolism and repair
- Unknown function

Figure 4- Categorization and frequency of the genes up- and down-regulated in wild-type cells grown in biofilm in relation to cells grown under planktonic conditions (A), and by Tec1 upon biofilm growth in *C. glabrata* (B), given by the *Δtec1* deletion mutant cells in relation to wild-type cells grown in biofilm, based on the biological process taxonomy of gene ontology (*P*-value<0.05).

suggesting that these cells might be experiencing nitrogen limitation. Considering the carbon and energy metabolism group, biofilm cells were down-regulating genes involved in the glycolytic pathway and up-regulating the glyoxylate cycle. This led us to speculate that these cells were also suffering from glucose deprivation, therefore using alternative carbon sources, as the 2-carbon products from β-oxydation of fatty acids. To confirm this, fatty acid metabolism pathway was analyzed and in fact, we could confirm that the biosynthesis of fatty acids is strongly down-regulated and its degradation is producing acetyl-CoA molecules that are likely to be used in the glyoxylate cycle for energy purposes.

Unveiling the effect of CgTec1 on the expression of the genes CgPWP5, CgAED2 and CgAWP13 encoding adhesins at different biofilm formation stages.

Among the 19 adhesion-related genes found to be activated by Tec1 during biofilm formation, there are six adhesin encoding genes: *CgAED1*, *CgAED2*, *CgAWP13*, *CgEPA9*, *CgEPA10* and *CgPWP5*. Interestingly, Tec1 was also found to control the expression of the TF encoding gene *CgEFG1*, another key biofilm formation regulator in *C. glabrata*[30]. This complex regulatory association controlling biofilm formation seems to be similar to what was reported in *C. albicans*, where Tec1 and Efg1 were shown to be mutually controlling each other's expression during biofilm development.[9]

Biofilm formation is initiated when planktonic cells find a surface where they can adhere and take advantage of the nutrients and other advantages.[31] The ability to adhere is a fast process, and even though our transcriptomic analysis gives us information about the adhesion-related genes expression of a mature biofilm, it would also be interesting to explore the transcriptomic variations of genes known to be important for biofilm formation in *C. glabrata* cells during all its developmental process. Thereupon, in an attempt to understand the variation of the level of transcripts at different time points of biofilm development, correspondent to some of the most important adhesins found in this study to be strongly regulated in biofilm cells by Tec1 TF, we chose *CgAED2*, *CgPWP5* and *CgAWP13* genes to be quantified by RT-qPCR. Gene expression was measured in wild-type and *Δtec1* cells after 6 h, 24 h and 48 h of biofilm development (**Figure 27**).

The expression of *CgAED2*, *CgPWP5* and *CgAWP13* was found to increase progressively in the wild-type strain, as the biofilm develops overtime. The results can be interpreted as the growing need for the biofilm cells to attach to each other, as it starts growing and developing the thick cell layers that are characteristic of this species. On the other hand, in the absence of *CgTEC1* expression levels are kept in relatively low levels, even decreasing slightly with time, suggesting that Tec1 is a crucial intervenient in regulating the adhesion phenomenon, gaining importance especially in the later stages of biofilm formation.

Tec1 activated CgAUR1, CgAED2 and CgSUR2 genes also play a role in biofilm development

Among the Tec1 activated genes up-regulated in biofilm cells, 8 were selected for further analysis for a role in *C. glabrata* biofilm formation, namely *AED2*, *PWP5*, *AWP13* genes encoding adhesins; *BMT1* and *BMT7* encoding beta-mannosyltransferases; *SUR2* gene, related to sphingolipid biosynthesis; *PUP1*, an uncharacterized gene encoding a mitochondria-localized protein and *CAGL0M10307g* (ortholog of *CaAUR1* and *ScAUR1* genes) related to drug resistance. As a first approach about the role of these genes on biofilm development, deletion mutants devoid of each of the mentioned genes, kindly provided by Dr. Hiroji Chibana, Chiba University, Japan, were grown to form biofilms in both RPMI and SDB media and compared to the wild-type KUE100 strain, grown in the same conditions. All deletion mutants tested, except for *Δpup1*, display decreased levels of biofilm formation, when compared to the wild-type population, when cultivated in RPMI medium (**Figure 6 A**). On the other hand, this observation is only confirmed for the *Δaur1* cells in SDB medium (**Figure 6 B**). Indeed, Aur1 appears to be a clear new determinant of biofilm formation in *C. glabrata*. *CgAUR1* (ORF *CAGL0M10307g*), however, remains uncharacterized in this species. In *S. cerevisiae*, Aur1 has been related to antifungal drug resistance, being determinant of Aureobasidin A (AbA) resistance, among other drugs. Genetic approaches allowed to identify *AUR1* gene as required for formation of inositolphosphorylceramide (IPC) in yeast, suggesting that this gene encodes part or all of the IPC synthase, which is essential for fungal sphingolipid biosynthesis.[32] Whether this is the role of *C. glabrata*'s Aur1 and whether IPC synthesis is required for biofilm formation remains uncharted.

Other genes that might deserve further investigations are the *CgAED2* and *CgSUR2* genes, since although they did not show a significant difference in the formed biofilms, compared to the wild-type strain, when grown in SDB medium, their absence was found to affect *C. glabrata* biofilm formation in RPMI medium.

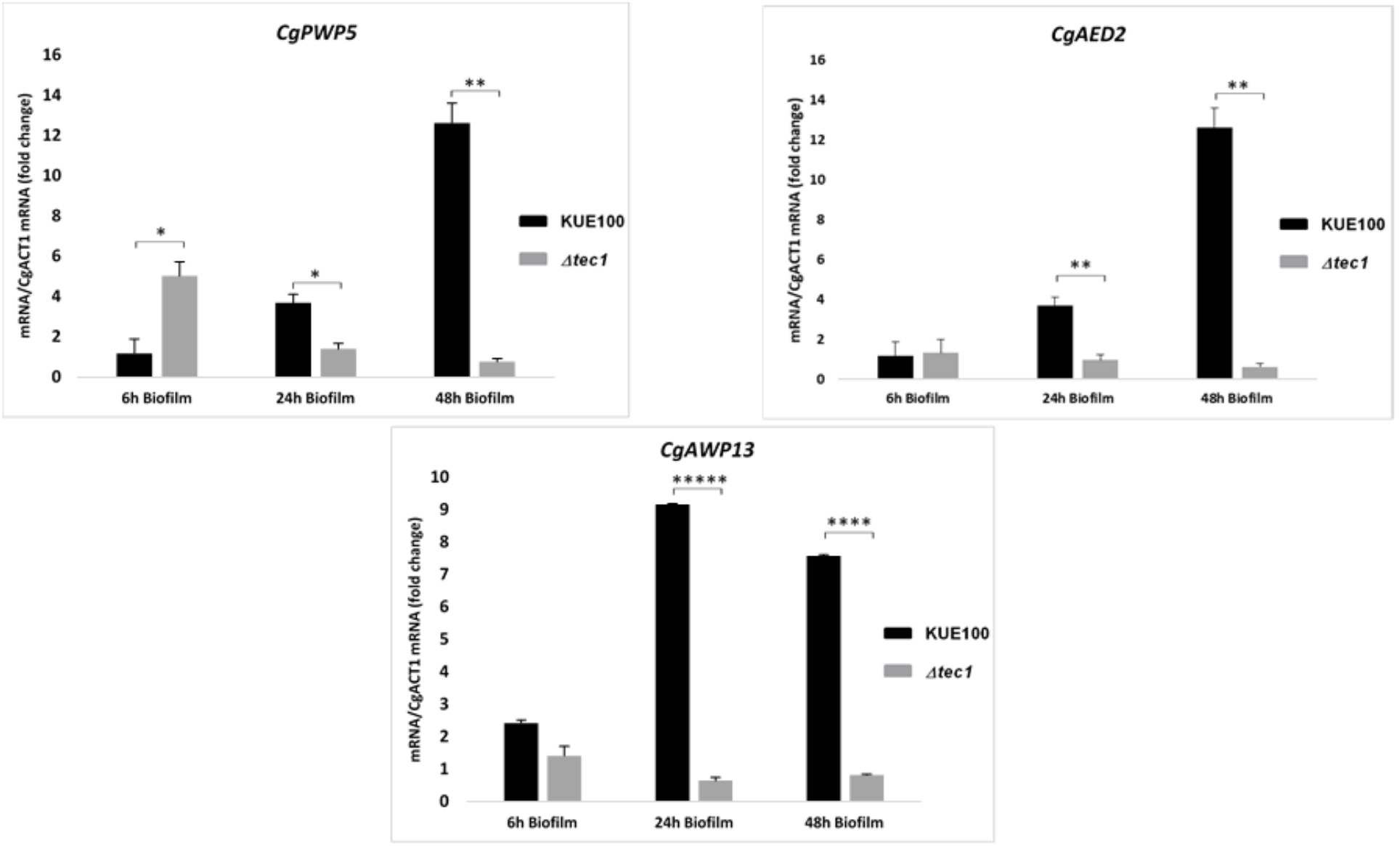

Figure 5- The deletion of the *CgTEC1* gene leads to a biofilm evolution-related decrease in the expression of the adhesion encoding genes *CgPWP5, CgAED2* and *CgAWP13*. Comparison of the variation of the *CgPWP5*, *CgAED2* and *CgAWP13* transcript levels in wild-type *C. glabrata* KUE100 cells and *Δtec1* deletion mutant cells, after 6 h, 24 h and 48 h of biofilm growth. The presented transcript levels were obtained by quantitative RT-PCR and are *CgPWP; CgAED2; CgAWP13 mRNA / CgACT1 mRNA* levels, relative to the values registered in wild-type cells after the chosen growth times. The indicated values are averages of at least three independent experiments. Error bars represent the corresponding standard deviations. **P*<0,05; ***P*<0,01; ****P*<0,001; *****P*<0,0001; ******P*<0,00001.

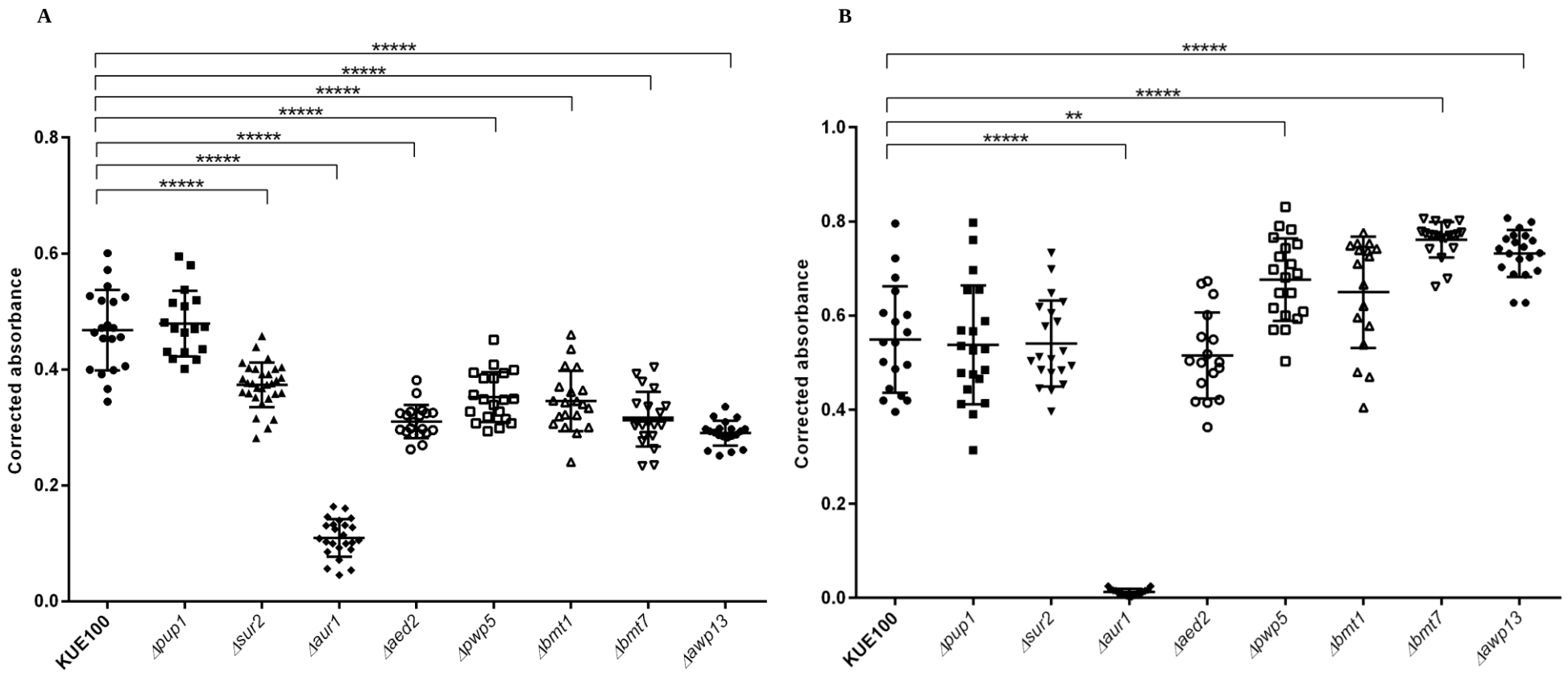

Figure 6- *CgAUR1* gene plays a role in adherence and biofilm development in RPMI (A) and SDB (B). Wild-type and the indicated deletion mutant cells were growth for 24 h in microtiter plates, after which cell viability was assessed based on PrestoBlue assay. A scatter dot plot representation of the data is shown, where each dot represents the level of biofilm formed in each sample. The average level of formed biofilm in at least 15 independent experiments is indicated by the black line, standard deviation being represented by the error bars. ****P*<0,001;*****P*<0,0001;******P*<0,00001.

Tec1 contributes to decreased ergosterol content in C. glabrata biofilms

Tec1 was found, through RNA-seq analysis, to regulate the expression of 7 ergosterol biosynthesis-related genes. Tec1 seems to act on decreasing ergosterol biosynthesis through the repression of *CgERG2*, *CgERG8* and *CAGL0L12364g* (ortholog of *CaERG10*) genes in biofilm cells. In an attempt to confirm these transcriptomic observations, and to uncover the final outcome of the changes in ergosterol biosynthesis genes found to be up- and down- regulated in biofilm cells, total ergosterol was extracted from biofilm and planktonic cells and quantified by HPLC. The ergosterol of the mutant cells *Δtec1* was compared to that of the wild-type KUE100 strain **(Figure 7).**

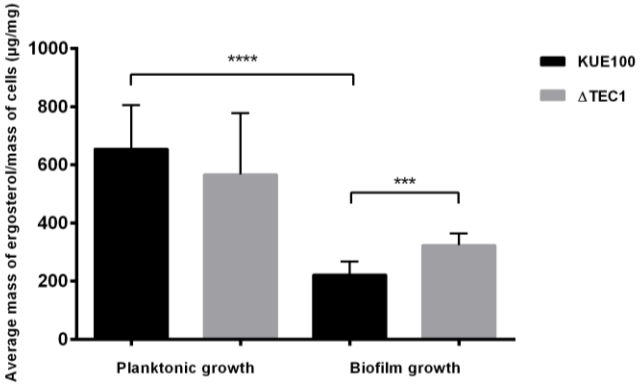

Figure 7- The ergosterol content in yeast cells is reduced when grown in biofilm and Tec1 is predicted to play a role in ergosterol content reduction in biofilm. Wild-type and single deletion mutant cells were harvested after 24 h of planktonic or biofilm growth and total ergosterol was extracted and quantified through HPLC. The displayed ergosterol content is the average of at least three independent experiments, standard deviation being represented by the error bars. ****P*<0,001; *****P*<0,0001.

From the ergosterol quantification results it is possible to ascertain that *C. glabrata* cells grown in biofilm suffer a significant reduction (almost 3-fold) of total ergosterol content. Additionally, Tec1 in fact acts on contributing for the reduction of ergosterol biosynthesis, as hypothesized by the RNA-seq data, as the deletion mutant cells displays a 31% increase on ergosterol content in biofilm cells. No significant differences were noted between both strains during planktonic growth.

In silico prediction of the CgTec1 recognition sequences, ligand binding sites and conserved domains

Once the recognized sequences targeted by Tec1 in *C. glabrata* are still unknown, a search for consensus sequences in the ORF promoter regions of each positively regulated gene, in planktonic and biofilm growth, was performed. By resorting to PathoYeastract database, these upstream regions were obtained and then, these were used in DREME, which yielded 2 consensus sequences for planktonic growth and 23 consensus sequences in respect to biofilm growth. Given the high number of predicted motifs, it is possible to narrow down the list by both excluding the ones containing TATA boxes, AAAs and TTTs regions, leaving 18 of 25 total predicted consensus. The investigation proceeded by searching for the Tec1 described binding motifs in *S. cerevisiae* and *C. albicans* (retrieved in Yeastract and PathoYeastract databases, respectively) and by searching for the number of Tec1 up-regulated genes, in our biofilm dataset, that included those consensus sequences in their up-stream region. This allowed us to observe the most significant part of the known Tec1 binding sites is CATTC, so by searching for this sequence in our retrieved motifs, **Table 1** was constructed with the most probable CgTec1 binding sequences.

Table 1- Tec1 predicted binding motif candidates.

Predicted Tec1 binding motifs
CAATGGBA
CAMATACA
CGATGSCC
GCGATGAS

FINAL DISCUSSION

In this work, Tec1 is strongly suggested to be a key player in biofilm development in *C. glabrata*. In the first part of this work, Tec1 was found to be required for adhesion to human vaginal epithelial cells and for biofilm formation in polystyrene surface. RNA-seq has been revolutionizing transcriptome profiling with deep-sequencing technologies. With this approach the transcriptome-wide changes of cells undergoing biofilm formation, in relation to planktonic cells, were assessed. This yielded a total of 3070 genes with altered expression, highlighting the multifactorial and complex nature of biofilm formation. The genes with altered expression levels were found to belong to multiple biological functions. Tec1 was found to up-regulate ¼ of the activated genes in biofilm cells, suggesting that this is a key regulator of biofilm-induced changes in *C. glabrata*. Indirectly, Tec1 also appears to inhibit ergosterol biosynthesis in biofilm cells. Interestingly, biofilm cells were found to contain decreased levels of ergosterol when compared to planktonically grown cells, which might be due to low oxygen levels felt by *C. glabrata* cells in the inner parts of the biofilm. The transcript levels of *CgAED2*, *CgPWP5* and *CgAWP13* genes, encoding adhesins, showed further that the action of Tec1 appears to be particularly crucial in later stages of biofilm formation. Among the Tec1 targets studied for a possible role in biofilm formation, Aur1, suggested to play a role in sphingolipid metabolism, was found to be crucial in this process. Further *in silico* analysis allowed the prediction of Tec1 protein structure and the search for conserved domains revealed the TEA/ATTS domain, which is described to be present in *C. albicans* and *S. cerevisiae* Tec1 orthologs, containing the predicted DNA binding residues. These observations encouraged the analysis of the promoter regions of Tec1 activated genes, which enabled the identification of possible binding sequences for this TF, similar to the ones already described in *S. cerevisiae* and *C. albicans*. The most probable were found to be "CAATGGBA", "CAMATACA", "CGATGSCC" and "GCGATGAS" (with B=C/G/T, M=A/C and S=G/C).

Many investigations have performed functional analysis of biofilm-induced genes. Perhaps the most elegant approach was undertaken by Nobile *et al.* (2012), who disclosed a transcriptional regulatory network underlying biofilm formation in *C. albicans*, extending the TF mutant screen. Tec1 was found in their study to be part of this complex regulatory network. In our work, Tec1 showed to control the expression of 1082 genes, with some genes found to encode for putative TFs. Therefore, Tec1 is proposed to participate in a possible regulatory network composed of several other TFs, regulating the biofilm formation phenomenon in *C. glabrata*.

Despite many open questions, the overall picture obtained with these results revealed the complexity and multifactorial nature of biofilm formation. The importance of Tec1 on regulating this phenomenon in *C. glabrata* is highlighted, placing this TF in a possible new drug target scenario, aiming to develop novel therapies that would prevent and overcome biofilm formation.

ACKNOLEDGMENTS

I thank my supervisor Professor Miguel Teixeira for all the support, guidance and motivation. I also thank to Professor Isabel Sá-Correia for giving me the chance to join the Biological Sciences Research Group to develop my master thesis work. For

the collaboration in the transcriptomic analysis herein accomplished, I thank Professor Geraldine Butler and her team, from University College of Dublin. For the supply of *Candida glabrata* mutants used in this work, I must thank Professor Hiroji Chibana, from University of Chiba, Japan. For the study developed in HPLC analysis of ergosterol levels, I also thank Professor Nuno Mira for his availability and assistance. To Professor Arsénio Fialho, PhD Dalila Mil-Homens and Msc Andreia Pimenta. I must thank the help in the adhesion assays, providing the human vaginal epithelium cells. This work was financially supported by Fundação para a Ciência e Tecnologia (FCT), contracts PTDC/BBB-BIO/4004/2014, PTDC/BII-BIO/28216/2017 and UID/BIO/04565/2013, and Programa Operacional Regional de Lisboa 2020, contract LISBOA-01-0145-FEDER-022231.

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