International Journal of Medical Microbiology 310 (2020) 151414

Contents lists available at ScienceDirect

International Journal of Medical Microbiology

journal homepage: www.elsevier.com/locate/ijmm

Effect of progesterone on *Candida albicans* biofilm formation under acidic conditions: A transcriptomic analysis

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ARTICLE INFO

Keywords:
Vulvovaginal candidiasis
Vaginal environment
Biofilm regulators
Expression profile

ABSTRACT

Vulvovaginal candidiasis (VVC) caused by *Candida albicans* is a common disease worldwide. A very important *C. albicans* virulence factor is its ability to form biofilms on epithelium and/or on intrauterine devices promoting VVC. It has been shown that VVC has a hormonal dependency and that progesterone affects virulence traits of *C. albicans* cells. To understand how the acidic environment (pH 4) and progesterone (either alone and in combination) modulate *C. albicans* response during formation of biofilm, a transcriptomic analysis was performed together with characterization of the biofilm properties. Compared to planktonic cells, acidic biofilm-cells exhibited major changes in their transcriptome, including modifications in the expression of 286 genes that were not previously associated with biofilm formation in *C. albicans*. The vast majority of the genes up-regulated in the acidic biofilm cells (including those uniquely identified in our study) are known targets of Sfl1, and consistently, Sfl1 deletion is herein shown to impair the formation of acidic biofilms (pH 4). Under the acidic conditions used, the presence of progesterone reduced *C. albicans* biofilm biomass and structural cohesion. Transcriptomic analysis of biofilms developed in the presence of progesterone led to the identification of 65 down-regulated genes including, among others, the regulator Tec1 and several of its target genes, suggesting that the function of this transcription factor is inhibited by the presence of the hormone. Additionally, progesterone reduced the susceptibility of biofilm cells to fluconazole, consistent with an up-regulation of efflux pumps. Overall, the results of this study show that progesterone modulates *C. albicans* biofilm formation and genomic expression under acidic conditions, which may have implications for *C. albicans* pathogenicity in the vaginal environment.

1. Introduction

Vulvovaginal candidiasis (VVC) affects millions of women every year and is considered to be an important public health problem. It is estimated that approximately 70–75 % of women will experience an episode of VVC in their lifetime (Sobel, 2007). Although VVC is not usually a life-threatening condition, the vaginal tract constitutes a putative access route to the bloodstream and therefore infections of this niche have the potential to result in disseminated infections, particularly in immunocompromised patients (Ascioglu et al., 2002). *Candida* species are opportunistic microbes of the vaginal commensal microflora, and under certain conditions, the usually symptomless colonization progresses into infection (Beigi et al., 2004). Despite an increased isolation of non-*Candida albicans Candida* species (NCAC) in women with VVC (Ahmad and Khan, 2009; Cetin et al., 2007), *Candida albicans* is still the most prevalent species (Amouri et al., 2011; Fan et al., 2008; Vermitsky et al., 2008). The ability of *C. albicans* to form biofilms is an important virulence factor as it confers unique phenotypic characteristics compared to its planktonic counterpart cells, including significant resistance to antifungal agents and host defense mechanisms while also providing protection against other physical and chemical stresses (Donlan and Costerton, 2002). In the vaginal environment, *Candida* species can form biofilms on the vaginal epithelium (Harriott et al., 2010) and also on intrauterine devices (IUDs) thereby promoting VVC (Chassot et al., 2008; Lal et al., 2008). The ability of *Candida* species to form biofilms in the vaginal environment is an important clinical problem, since the recalcitrant nature of biofilms to currently used antifungals prevents definitive eradication of these microbes from the vaginal lumen thus contributing to the recurrence of VVC (Harriott et al., 2010).

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https://doi.org/10.1016/j.ijmm.2020.151414
Received 4 May 2019; Received in revised form 8 January 2020; Accepted 25 February 2020

The development of VVC has been associated with the disturbance of the hormonal vaginal environment resulting from behavioral or other host-related factors such as pregnancy, hormone replacement therapy, and use of oral contraceptives or IUDs (Sobel, 2007). It is thought that progesterone contributes to VVC development by stimulating the production of glycogen by epithelial cells (Dennerstein and Ellis, 2001; Špaček et al., 2007) and inhibiting certain traits of the innate and adaptive immune response (Keller et al., 2007). Besides these effects on the host it has been also shown that progesterone has direct effects on the physiology of *Candida* cells. Several *Candida* species such as *C. albicans*, *Candida guilliermondii*, *Candida krusei*, *Candida parapsilosis* and *Candida tropicalis* have corticosterone receptors with high affinity for progesterone (Loose et al., 1983; Skowronski and Feldman, 1989). A transcriptional survey of the effect of progesterone on *C. albicans* planktonic cells identified activation of stress response pathways, including the induction of genes involved in host immune and drugs response (Banerjee et al., 2007). Banerjee et al. (2007) also reported that progesterone decreases *C. albicans* drug susceptibility and found higher MIC for fluconazole and ketoconazole when *C. albicans* planktonic cells are exposed to progesterone. In a previous study, we have shown (Alves et al., 2014) that progesterone, at pregnancy levels, reduces the ability of *C. albicans* strains to form biofilms. This unexpected finding fostered the present work in which we aimed to deepen the current understanding on how progesterone modulates the process of *C. albicans* biofilm formation at the vaginal acidic pH, something that has not been examined before and that is essential for a full understanding of the pathogenesis of this species in the acidic vaginal tract.

2. Material and methods

2.1. Strains and growth conditions

The reference strain *C. albicans* SC5314 was the main strain used in this work. Additionally, some experiments were carried out using *C. albicans* mutant strains and their respective parent. All the strains are listed in Table 1.

For each experiment the strains were subcultured on Sabouraud dextrose agar medium (SDA; Merck, Darmstadt, Germany) for 48 h at 37 °C. An inoculum from the SDA plate was suspended in 25 mL of Sabouraud dextrose broth (SDB; Merck, Darmstadt, Germany) and incubated for 18 h under agitation (120 rev/min) at 37 °C. Under these conditions the cells were found to be at the beginning of stationary phase. After incubation, the cells were harvested by centrifugation at 3000 g for 10 min at 4 °C and washed twice with 15 mL of Phosphate Buffered Saline (PBS; pH 7). Pellets were suspended in Roswell Park Memorial Institute medium (RPMI) (Sigma-Aldrich, St Louis, MO, USA), buffered with MOPS (3-(N-morpholino) propane sulfonic acid) and adjusted to pH 4 with HCl. The cell density was further adjusted to 1×10^7 cells/mL for each experiment, using a Neubauer haemocytometer (Marienfeld, Lauda-Königshofen, Germany).

2.2. Effect of progesterone on C. albicans planktonic growth

A stock solution of progesterone (Sigma-Aldrich, St Louis, MO, USA) at 10 mM was prepared on ultrapure water and stored at −20 °C to be used in all experiments. *C. albicans* SC5314 cells from the pre-inoculum were cultivated in RPMI at pH 4, either supplemented or not with 2 μM of progesterone (Alves et al., 2014). Cultures of *C. albicans* cells (1×10^7 cells/mL with and without progesterone) were placed in 25 mL Erlenmeyer flasks, maintained at 37 °C with agitation (120 rev/min) and the increase in optical density at 600 nm was measured over time using a microtiter plate reader (Bio-Tek Synergy HT, Izasa, Winooski, VT, USA). The optical density of the initial cultures was 0.01. The results were presented as optical density over 30 h of growth.

2.3. Effect of progesterone on C. albicans biofilm formation

2.3.1. Biofilm formation

To study the effect of progesterone on biofilm formation, biofilms were developed in presence and absence of progesterone as described by Alves et al. (2014). Briefly, suspensions (1×10^7 cells/mL) of *C. albicans* SC5314 cells from the pre-inoculum were prepared in RPMI at pH 4, either supplemented or not with 2 μM of progesterone, and placed into wells of 96-wells polystyrene microtiter plates (Orange Scientific, Braine-l`Alleud, Belgium) (200 μL per well). The plates were incubated for 24 h at 37 °C under agitation (120 rev/min). After incubation, the medium was aspirated and non-adherent cells were removed by washing the biofilms with 200 μL of PBS. The analyses of the biofilms were performed in triplicate (biofilms formed from the same pre-inoculum) and in three independent assays (biofilms formed from pre-inoculums independently prepared).

2.3.2. Biofilm cultivable cells determination

The number of cultivable cells on biofilms grown with and without progesterone was determined by measuring colony forming units (CFUs) (Silva et al., 2009). Briefly, biofilms were scraped from the microtiter plates wells with 200 μL of PBS and the suspensions were vigorously vortexed for 2 min to disaggregate cells. Serial dilutions in PBS of the resuspended biofilms were plated on SDA and incubated for 24 h at 37 °C. After incubation, the number of colonies on the SDA plates was counted and the results were presented as total of colony forming units (CFUs) per unit area of microtiter plate well (Log (CFUs/mL)/cm^2).

2.3.3. Biofilm total biomass quantification

Biofilms' total biomass, formed with and without progesterone, were quantified using crystal violet (CV) staining methodology (Silva et al., 2009). Biofilms were fixed on the microtiter plates wells with 200 μL of methanol, which was removed after 15 min. The microtiter plates were allowed to dry at room temperature and biofilms were stained by the addition of 200 μL of CV (1%, v/v) to each well. After 5 min, CV was removed and biofilms were washed twice with sterile water to remove the excess of stain. Finally, 200 μL of acetic acid (33 %, v/v) were added to each well to release and dissolve the stain and the absorbance of the obtained solutions was measured in a microtiter plate reader (Bio-TekSynergy HT, Izasa, Winooski, VT, USA) at 570 nm. The results were presented as absorbance per unit area of microtiter plate well (Absorbance CV/cm^2).

2.3.4. Biofilm metabolic activity determination

A XTT reduction assay (Hawser, 1996) was used to determine the metabolic activity of the biofilms formed in the presence and absence of

Table 1
Candida albicans strains used in this study.

Strain name	Parent	Relevant genotype	Reference
SC5314		Prototrophic	Lab collection
SN76	SC5314	*arg4Δ/arg4Δ, his1Δ/his1Δ, ura3Δ-iro1Δ::λimm^{434}/ura3Δ-iro1Δ::λimm^{434}*	(Noble and Johnson, 2005)
sfl1Δ/sfl1Δ	SN76	*sfl1Δ::ARG4/sfl1Δ::HIS1*	(Znaidi et al., 2013)
tec1Δ/tec1Δ	SN76	*tec1Δ::ARG4/tec1Δ::HIS1*	(Znaidi et al., 2013)
brg1Δ/brg1Δ	SN76	*brg1Δ::ARG4/brg1Δ::HIS1*	(Znaidi et al., 2013)

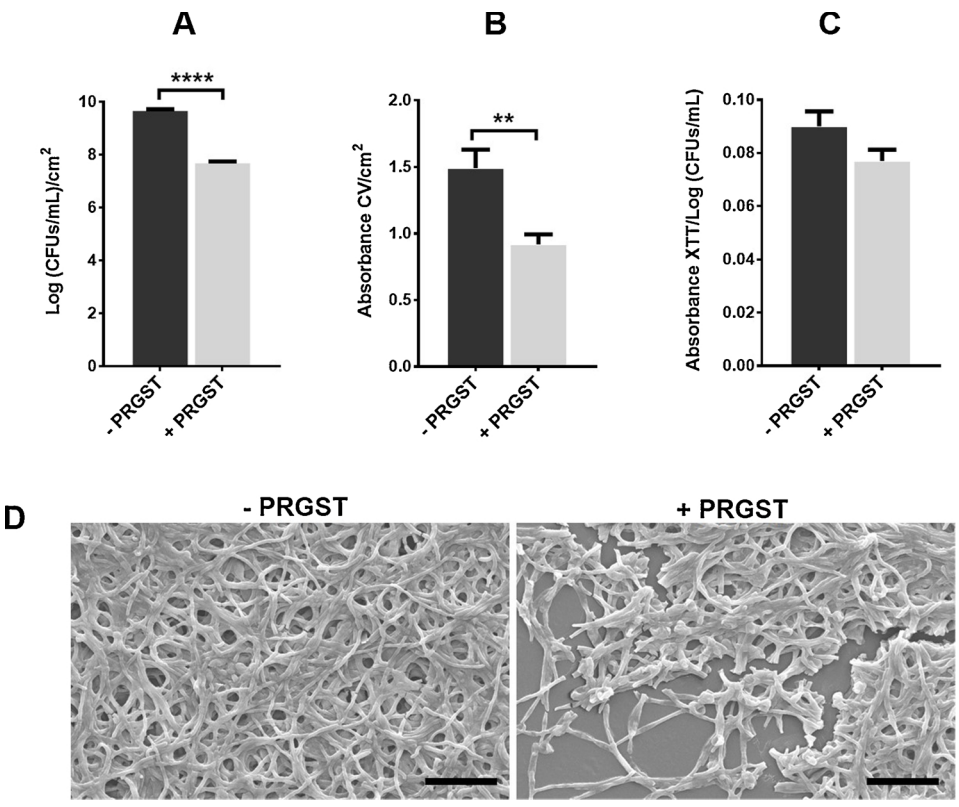

Fig. 1. Effect of progesterone on *C. albicans* biofilm formation. **(A)** Cell cultivability determination [Log (CFUs/mL)/cm^2], **(B)** total biomass quantification (Absorbance CV/cm^2), **(C)** metabolic activity measurement (Absorbance XTT/Log (CFUs/mL)) and **(D)** scanning electron microscopy images of *C. albicans* SC5314 biofilms grown 24 h in RPMI at pH 4 in absence (-PRGST) or presence of 2 μM of progesterone (+PRGST). Error bars represent standard deviation. Asterisks represent statistical difference between the conditions (**** *p*-value ≤0.0001; ** *p*-value ≤0.01). Original magnification of panel the images of panel D was x 1000 and the scale bars correspond to 20 μm.

progesterone. A 200 μL aliquot of a solution containing 100 μg/μL of XTT (2, 3-(2-methoxy-4-nitro-5-sulfophenyl)-5-[(phenylamino) carbonyl]-2H-tetrazolium hydroxide) (Sigma-Aldrich, St Louis, MO, USA) and 10 μg/μL of PMS (phenazine methosulfate) (Sigma-Aldrich, St Louis, MO, USA) was added to wells with developed biofilms. The plates were incubated for 3 h in the dark, at 37 °C under agitation (120 rev/min). Then, 150 μL of supernatant of each well were transferred to a new microtiter plate reader (Bio-Tek Synergy HT, Izasa, Winooski, VT, USA) and the colorimetric changes were measured at 490 nm. The absorbance values were normalized with respect to the CFUs and are presented as Absorbance XTT/Log (CFUs/mL).

The metabolic activity of planktonic cells grown as described above, in presence and absence of progesterone, was also determined at specific time points up to 24 h. In this case 50 μL aliquot of XTT/PMS was added to 200 μL of cell suspension and the metabolic activity was analyzed as described for biofilms.

2.3.5. Biofilm structure analysis

The structure of *C. albicans* biofilms formed with and without progesterone was analyzed by scanning electron microscopy (SEM) (Alves et al., 2014). Biofilms were formed as described above, except on 24-well polystyrene microtiter plates (Orange Scientific, Braine-l'Alleud, Belgium) (1 mL of cell suspension per well). Developed biofilms were dehydrated with ethanol (using 70 % ethanol for 10 min, 95 % ethanol for 10 min and 100 % ethanol for 20 min), air dried for 20 min and placed in a desiccator until analysis. Before observation, the base of the wells was removed and mounted onto aluminum stubs, sputter coated with gold. Biofilms were then imaged with an S-360 scanning electron microscope (Leo, MA, Cambridge, USA).

2.3.6. Biofilm cells antifungal susceptibility testing

The susceptibility of *C. albicans* biofilm cells to fluconazole was tested using the reference protocol for broth microdilution antifungal susceptibility testing of yeasts, according to the Clinical and Laboratory Standards Institute methods (CLSI, 2017). Biofilms developed as described above, in presence and absence of progesterone, were scraped from the wells with PBS and the suspensions were vigorously vortexed for 2 min and centrifuged at 5000 g for 5 min at 4 °C. Pellets were resuspended in RPMI at pH 4 and the cell suspensions were used to the antifungal susceptibility tests and minimal inhibitory concentrations determination (MICs), according to the reference method.

2.4. Transcriptomic analysis

The effect of progesterone on the transcriptome of *C. albicans* biofilms was assessed using species-specific DNA microarrays (Bernardo et al., 2017). For this, the transcriptomes of *C. albicans* SC5314 cells present in biofilms grown for 24 h in absence and presence of progesterone (2 μM) were compared with the transcriptome of planktonic cells cultivated for the same time in hormone-free RPMI medium (pH 4). The experimental setups used to cultivate the cells in planktonic and biofilm life styles were the same as described above and using 24-well polystyrene microtiter plates (1 mL per well). Developed biofilms obtained after 24 h of cultivation in presence and absence of progesterone were scraped from the plates with PBS and the suspensions were sonicated (Ultrasonic Processor, Cole-Parmer, IL, USA) for 30 s at 30 W, to separate the cells from the biofilm matrix (Silva et al., 2009). Then, all cell suspensions (planktonic and biofilm-forming cells) were centrifuged at 3000 g for 10 min at 4 °C, the supernatants were rejected and pellets were used for RNA extraction.

2.4.1. RNA extraction

Total RNA extraction was performed using the RiboPure – Yeast Kit (Life Technologies, Carlsbad, CA, USA), according to the manufacturer's protocol. RNA concentration and purity were determined by spectrophotometry and its integrity was confirmed using an Agilent 2100 Bioanalyzer with an RNA 6000 Nano Assay (Agilent Technologies, Santa Clara, CA, USA).

Table 2
Subset of genes found to be biofilm-regulated (up- and down) in *C. albicans* cells cultivated 24 h at pH 4. A subset of genes whose expression was found to increase or decrease (above or below 2-fold) in *C. albicans* SC5314 biofilms grown 24 h in RPMI at pH 4, in comparison with the transcript levels registered in planktonic cells cultivated in the same conditions, was selected and are herein shown, while the full list is available on supplementary Table S2. Genes whose transcription was found to be biofilm-induced or –repressed specifically at the acidic conditions used in this study, are highlighted in grey and among them are highlighted in bold those previously described as essential to biofilm formation. The biological function indicated is based on the information available at Candida Genome Database.

Up-regulated genes		
Gene	Biological function	mRNAbiofilm / mRNAplanktonic
ALS1	Cell-surface adhesin	20.17
ECE1	Candida lysin	18.12
CAN2	Basic aminoacid permease	16.91
SOD5	Cu-containing superoxide dismutase	15.01
C1_13130C_A	Putative histidine permease	12.59
COX3B	Cytochrome c oxidase involved in mitochondrial respiration	10.97
HGT2	Putative MFS glucose transporter	10.57
HYR1	Hyphal cell wall protein involved in biological adhesion	9.87
NAD1	NADH dehydrogenase involved in mitochondrial respiration	9.69
NAD3	NADH dehydrogenase involved in mitochondrial respiration	9.49
C2_10160W_A	Secreted protein	9.30
HGT1	High-affinity MFS glucose transporter	9.18
DIP5	Dicarboxylic aminoacid permease	8.97
ATP6	Subunit 6 of the F0 sector of mitochondrial F1F0 ATP synthase	7.84
PRA1	Cell surface protein that sequesters zinc from host tissue	5.88
QDR1	Putative antibiotic resistance transporter	5.71
C7_00490C_A	Putative AdoMet-dependent proline methyltransferase	2.93
NPL3	Putative RNA-binding protein	2.63
CSA2	Extracellular-associated protein involved in iron assimilation	2.58
C1_00160C_A	Putative nucleolar protein	2.57
ALS5	ALS family adhesin	2.38
NDT80	Meiosis-specific transcription factor	2.28
WOR1	Transcription factor of white-opaque phenotypic switching	2.16
Down-regulated genes		
Gene	Biological function	mRNAbiofilm / mRNAplanktonic
DUR3	High affinity spermidine transporter	0.05
MEP2	Ammonium permease	0.09
JEN2	Dicarboxylic acid transporter	0.11
C2_00180C_A	Predicted uricase	0.15
C1_01630W_A	Putative mitochondrial complex I intermediate-associated protein	0.15
FDH1	Formate dehydrogenase	0.17
C1_07160C_A	Protein conserved among the CTG-clade	0.17
CWH8	Putative dolichyl pyrophosphate (Dol-P-P) phosphatase	0.18
HSP30	Putative heat shock protein	0.18
LAP3	Putative aminopeptidase	0.18
CTN1	Carnitine acetyl transferase	0.18
C2_01660C_A	Unknown function	0.18
CCP1	Putative Cytochrome-c peroxidase	0.18
MDH1-1	Predicted malate dehydrogenase precursor	0.18
C5_03770C_A	Putative formate dehydrogenase	0.18
C5_03710C_A	Unknown function	0.18
DUR1,2	Urea amidolyase involved in response to abiotic stimulus	0.19
ADH2	Alcohol dehydrogenase;	0.19
PGA13	GPI-anchored cell wall protein involved in cell wall synthesis	0.19

2.4.2. Microarray analysis

cDNA synthesis, hybridization and scanning were performed using protocols similar to those described previously (Rossignol et al., 2007), with the exception of that hybridization was carried out using an Agilent hybridization oven at 65 °C for 17 h at 100 rpm. In brief, 6 μg of total RNA was incubated with 1.4 μg of anchored Oligo$(dT)_{20}$ primer (Invitrogen, Carlsbad, CA, USA) in a total volume of 18.5 μL for 10 min at 70 °C. First-strand buffer (Invitrogen, Carlsbad, CA, USA), 0.5 mM dATP, dTTP, and dGTP; 50 μM dCTP; 10 mM dithiothreitol; 2 μL Superscript III reverse transcriptase (Invitrogen, Carlsbad, CA, USA); and 2 μL Cy3-dCTP or Cy5-dCTP (Amersham, PA53021 and A55021) were added to a total volume of 40 μL, incubated at 42 °C for 2 h followed by 1 h at 42 °C with an additional 1 μL of Superscript III. RNA was degraded by addition of 1 μL of RNase A at 50 μg/mL and 1 μL of RNase H at 1 unit/μL and incubated at 37 °C for 30 min. The labelled cDNAs were purified using a QIAquick PCR purification kit (Qiagen, Hilden, Germany), using a modified protocol. Samples were prepared for hybridization using Agilent's Two-Color Microarray-Based Gene expression (Quick Amp labelling protocol) and the Gene Expression Hybridization Kit. 10 μL each Cy3-labeled and Cy5-labeled cDNA were used per array (8 × 15 k, design ID 065138), in a total volume of 50 μL. Hybridized microarrays were scanned with an Axon 4000B scanner (Axon Instruments, Union City, CA, USA) and data were acquired with Gene Pix Pro 5.1 software (Axon Instruments, Union City, CA, USA). Data was analyzed using the LIMMA package in Bioconductor (www.bioconductor.org). Three biological replicates and three technical replicates were performed. Lowess normalization and background correction were applied to each array separately, and quantile normalization was used to allow log ratios to be compared across arrays. Only genes exhibiting $\log_2$FC > 1.0 and having an associated *p*-value below 0.01 were selected for further analysis. The data was submitted to the Array Express database with the reference E-MTAB-8672.

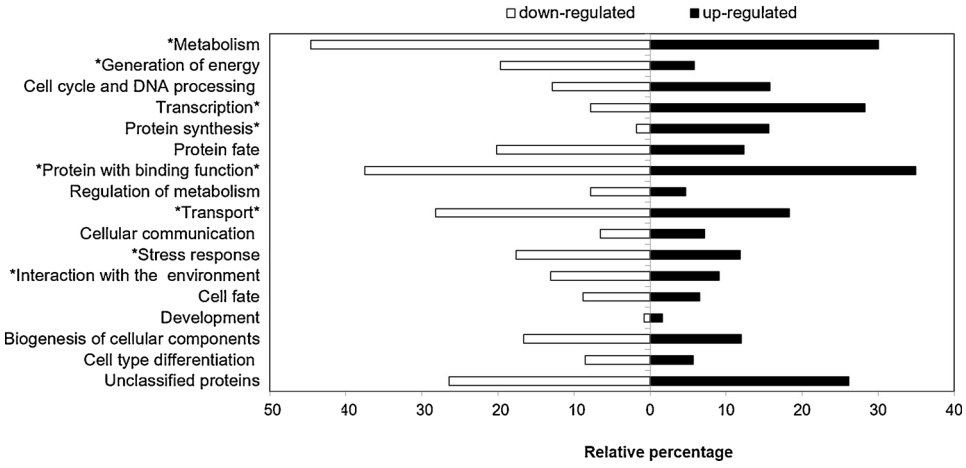

Fig. 2. Functional distribution of genes found to be biofilm-regulated in *C. albicans* cells cultivated for 24 h in RPMI at pH 4. The genes found to be up- or down-regulated in *C. albicans* SC5314 biofilms grown 24 h in RPMI at pH 4, in comparison with the levels found in planktonic cells cultivated in the same conditions, were clustered according to their biological function, using MIPS Functional Catalogue database (black and white bars correspond to up- and down-regulated genes, respectively). The percentages shown correspond to the number of genes included in each functional class compared to the total number of up- or down-regulated genes. Functional categories considered significantly enriched in the datasets (p-value < 0.001; taking into consideration the entire genome of *C. albicans* SC5314 reference strain) are indicated with asterisk (asterisk before or after the class denomination means enrichment among down- or up-regulated genes, respectively).

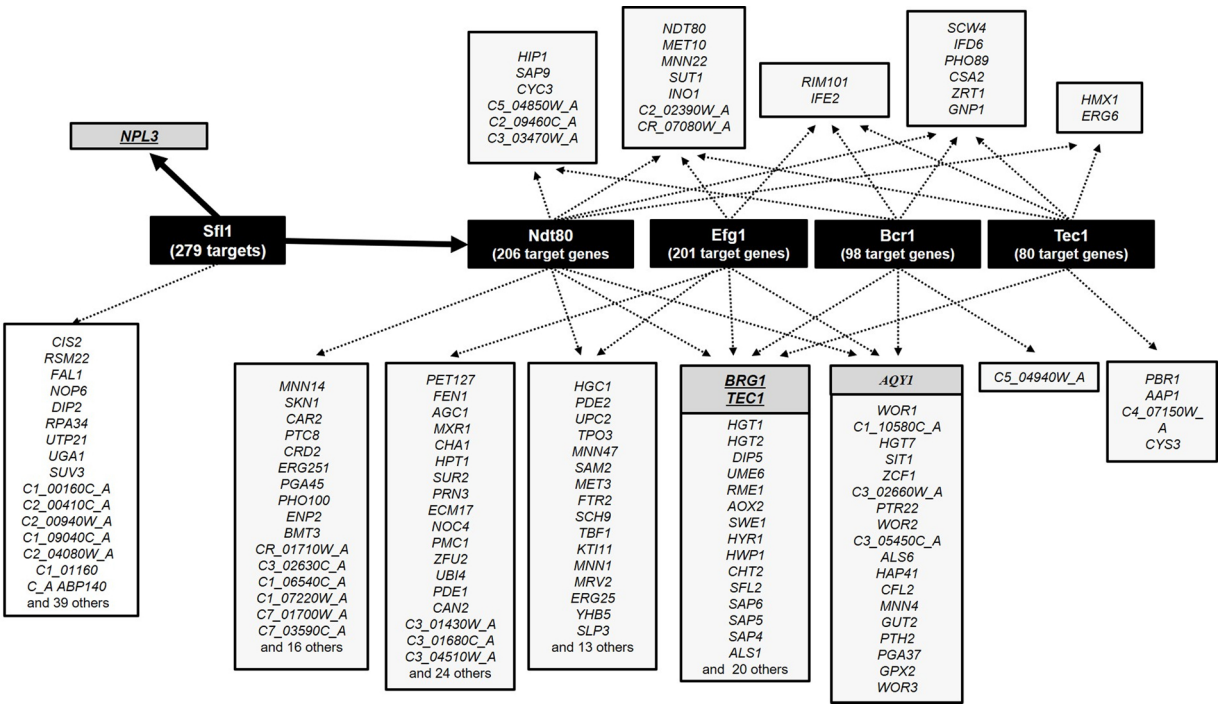

Fig. 3. Proposed transcriptional regulatory network underlying the control of *C. albicans* biofilm cells after 24 h of cultivation in RPMI at pH 4. The genes found to be up-regulated in *C. albicans* SC5314 biofilms formed after 24 h of cultivation in RPMI at pH 4, in comparison with the levels attained in planktonic cells, were clustered according with the existence of documented regulatory associations with transcription factors mediating the control of transcriptome-wide alterations related with biofilm formation, according with the information available on the PathoYeastract database. The number of documented targets attributed to each transcription factor is indicated in brackets inside the black boxes. Only genes having a biological function related with adhesion and/or biofilm formation (or described to be up-regulated under these conditions) were selected for this analysis, being identified in grey boxes those genes whose deletion was described to abolish biofilm formation. The results that gave rise to this figure are fully detailed in supplementary Table S3.

2.5. Measurement of gene transcription based on quantitative real-time PCR

In order to validate some of the results obtained in the microarray analysis, the expression profile of a set of specific genes (*TEC1*, *CRZ2* and *CDR1*) was obtained using quantitative real-time PCR (qRT-PCR). The experimental conditions used to cultivate *C. albicans* SC5314 cells and to obtain RNA were the same as those described above for the transcriptomic analysis. Then, 0.5 μg of total RNA collected from each sample was used to obtain the complementary DNA (cDNA), using the iScriptcDNA Synthesis Kit (Bio-Rad, Hercules, CA, USA) according to the manufacturer's instructions. cDNA synthesis was performed at 70 °C for 5 min followed by 42 °C for 1 h and lastly 5 min at 95 °C to stop the reaction. Approximately 125 ng of the synthesized cDNA were used for the qRT-PCR. qRT-PCR (CF X96 Real-Time PCR System; Bio-Rad, Hercules, CA, USA) was used to determine the relative levels of mRNA transcripts, with the transcript level of *ACT1* mRNA used as an internal control. Primers for the target genes and *ACT1* were designed using Primer3 web software and their sequences are provided in supplementary Table S1. Full-length gene sequences were obtained from the *Candida* Genome Database (www.candidagenome.org) (Skrzypek et al., 2010). The sequence of each primer was compared to the *Candida* genome database using BLAST (Altschul et al., 1997), to confirm their

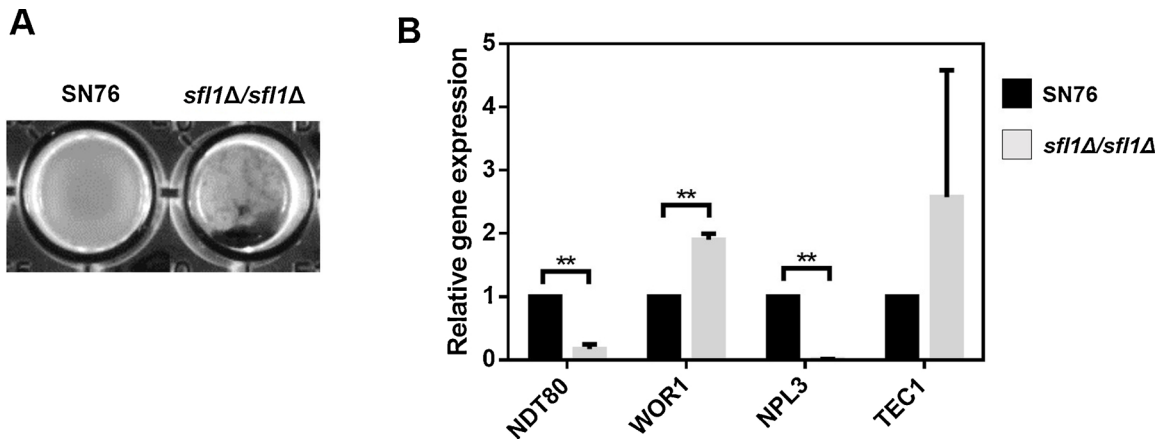

Fig. 4. (A) Sfl1 is required for maximal *C. albicans* biofilm formation under acidic conditions (pH 4); *C. albicans* homozygous mutant strain lacking *SFL1* (*sfl1Δ/sfl1Δ*) and the respective parental strain (SN76), were cultivated in RPMI at pH 4 for 24 h. The biofilms formed under these conditions were photographed and are shown. **(B)** Effect of Sfl1 expression in transcription of *NDT80*, *WOR1*, *NPL3* and *TEC1* in biofilm cells after 24 h of cultivation in RPMI under the same conditions as those used in the phenotypic assays shown in panel A. Error bars represent standard deviation. Asterisks represent statistical difference between the results of the mutant and parent strain. (** *p*-value ≤0.01).

specificity. The specificity of each primer pair for its corresponding target gene was also confirmed, applying a PCR to genomic DNA extracted from *C. albicans* SC5314 planktonic cells, using the various primer pairs. Then, qRT-PCR was performed using reaction mixtures consisted of SsoFast EvaGreen Supermix (Bio-Rad, Hercules, CA, USA), dH_2O (Cleaver Scientific Ltd, Warwickshire, UK), cDNA samples and the primer pair respective to the target gene (50 μM). Negative controls (dH2O) and non-reverse transcriptase controls (NRT) were also included in each run. qRT-PCR was performed at 98 °C for 2 min in the initial denaturation step, followed by denaturation at 98 °C for 5 s and primer annealing at 57 °C for 5 s, during 40 cycles. In each cycle a melting curve was generated, running a dissociation stage at 60 °C, to verify the amplification product specificity. Control samples were included on each plate to ensure that multiple plates could be compared. The Ct value of each sample was determined and the relative gene expression levels calculated using the ΔCt method (Livak and Schmittgen, 2001), being normalized with the internal control gene ($Ct_{average}$ = 23.02 ± 1.35; three biological replicates). Each reaction was performed in quadruplicate and mean values of relative expression were determined for each gene.

2.6. Experiments using C. albicans mutant strains

In order to investigate the role of some genes (*SFL1, TEC1*and *BRG1*) suggested by our microarray analysis to be important to the biofilm formation in the specific conditions used in this study (pH 4 and presence of progesterone), *C. albicans* biofilms of homozygous null mutants were formed. Mutant and respective parental strains (auxotrophs) used for these experiments are listed in Table 1. Biofilms were developed as previously described for *C. albicans* SC5314 using RPMI at pH 4 supplemented or not with progesterone. Biofilms grown for 24h were washed with PBS and imaged with ChemiDoc Image System (Bio-Rad, Hercules, CA, USA). Additionally, gene expression of mutants biofilm cells was analyzed by qRT-PCR. For that, biofilm formation, RNA extraction and qRT-PCR procedures were performed as previously described for qRT-PCR analyses of *C. albicans* SC5314. Primers for the target genes were designed using Primer3 web software and their sequences are provided in supplementary Table S1.

2.7. Statistical analysis

Results of biofilm biomass, cells cultivability, metabolic activity and gene expression (qRT-PCR) were statistically analyzed using t tests implemented in GraphPad Prism 6 software. In all the experiments three biological and technical replicates were performed. The statistical tests were performed with a confidence level of 95 %.

3. Results and discussion

3.1. Effect of progesterone on C. albicans planktonic growth and biofilm formation

To obtain a clearer picture of the effects exerted by progesterone on the physiology of *C. albicans* we examined how the presence of this hormone affects growth of the yeast cells, in either planktonic or biofilm-forming conditions. The assays were performed in RPMI medium at pH 4 in order to mimic the acidic vaginal environment (in the range of 3.6–4.5) (Boskey, 2001). The medium was supplemented with 2 μM of progesterone, which is the highest concentration reported in the plasma of pregnant women in the third trimester (Alves et al., 2014), as the hormonal disturbance promoted by pregnancy is considered an important risk factor for VVC development (Sobel, 2007). In planktonic conditions, the concentration of progesterone used had only a minor effect on the growth rate of *C. albicans* SC5314 cells, comparatively to what was observed in unsupplemented RPMI medium (supplementary Fig. S1). However, the presence of progesterone reduced the ability of *C. albicans* SC5314 to form biofilms, resulting in a statistically significant decrease on cell cultivability (*p*-value ≤0.0001) and total biomass (*p*-value ≤0.01) (Fig. 1A and B), compared to biofilms formed in the absence of the hormone. Specifically, we observed a decrease in the number of cultivable cells of approximately 2 orders of magnitude [Log (CFUs/mL)/cm^2] (Fig. 1A) and a decrease in biofilm biomass of approximately 20 % (Fig. 1B). These results are consistent with those reported by Alves et al. (2014). It is important to stress that the study of Alves et al. (2014) was undertaken using two *C. albicans*strains (ATCC 90028 and 558234), a reference strain from the American Type Culture Collection and a vaginal isolate, respectively, different from the one used in this study. This lead us to conclude that the inhibitory effect of progesterone on biofilm formation by *C. albicans* is independent of the genetic background of the strains used.

The effect of progesterone on the metabolic activity of C. *albicans* biofilm's cells was also evaluated, using XTT reduction assay. The results obtained (Fig. 1C) show a slight decrease in the metabolic activity of biofilm cells cultivated in the presence of progesterone, compared to the absence of the hormone; however, this difference is not statistically significant (*p*-value > 0.05). Progesterone also did not decrease metabolic activity of planktonic cells (data not shown). The effect exerted by progesterone on the structure and morphological characteristics of the

Table 3
Subset of progesterone-responsive genes in *C. albicans* SC5314 biofilms grown 24 h at pH 4. Was selected and listed here a subset of *C. albicans* SC5314 genes found be up-regulated in biofilms grown 24 h in RPMI at pH 4, in the presence (+ PRG) and/or absence(- PRG) of 2 μM of progesterone and whose expression differed more than 40 % between the two biofilm-forming conditions. This table includes a subset of genes found to be up-regulated in both conditions, but showing a stronger induction in the presence of the hormone (group *i*) and a subset of genes strongly up-regulated in biofilms without progesterone than on its presence (group *ii*). The full list of progesterone-responsive genes is available on supplementary Table S5. The biological function indicated is based on the information available at Candida Genome Database.

Group *i)*

Gene	Biological function	mRNA +PRG	mRNA -PRG	mRNA +PRG/mRNA -PRG
ERG6	Protein involved in ergosterol biosynthesis	12.65	5.22	2.43
CDR2	Multidrug transporter of ABC superfamily	3.15	1.35	2.33
PGA31	Cell wall protein involved in response to drug	10.18	4.85	2.10
PGA45	Putative GPI-anchored cell wall protein	20.17	9.66	2.09
CDR1	Multidrug transporter of ABC superfamily	3.15	1.53	2.06
POT1	Putative peroxisomal 3-oxoacyl Co Athiolase	2.61	1.30	2.00
C3_02870C_A	Unknown function	4.74	2.49	1.90
GAP2	General amino acid permease	2.14	1.18	1.82
ATO1	Putative transmembrane protein	7.62	4.31	1.77
C7_02260W_A	Unknown function	3.23	1.85	1.74
INO1	Inositol-1-phosphate synthase	47.93	28.05	1.71
FAS2	Alpha subunit of fatty-acid synthase	2.43	1.44	1.68
C2_05980C_A	Putative acyl-CoA hydrolase activity	3.17	1.89	1.68
PTH2	Putative cAMP-independent regulatory protein	3.60	2.19	1.64
AGP3	Putative serine transporter	5.12	3.20	1.60
C4_00250W_A	Unknown function	3.48	2.18	1.59
FAT1	Predicted enzyme of sphingolipid biosynthesis	2.12	1.33	1.59
GIT2	Putative glycerol phosphoinositol permease	5.04	3.19	1.58
CSA2	Protein involved in iron assimilation	4.07	2.58	1.58

Group *ii)*

Gene	Biological function	mRNA +PRG	mRNA -PRG	mRNA +PRG/mRNA -PRG
COX3B	Cytochrome c oxidase	1.54	10.97	0.14
NAD3	NADH dehydrogenase	1.38	9.49	0.15
ATP9	ATP synthase required for ATP synthesis	1.15	6.63	0.17
ATP6	ATP synthase required for ATP synthesis	1.52	7.84	0.19
NAD6	NADH dehydrogenase	1.69	8.54	0.20
COX2	Cytochrome c oxidase	1.76	8.56	0.21
WOR3	Transcription factor	0.85	2.63	0.32
SUT1	Zn2Cys6 transcription factor	0.96	2.16	0.44
BRG1	Transcription factor	0.89	2.01	0.44
RME1	Zinc finger protein; putative meiosis control	2.20	4.55	0.48
PRN3	Protein similar to pirin; unknown function	1.53	2.84	0.54
PRN1	Protein similar to pirin; unknown function	1.61	2.90	0.56
TEC1	TEA/ATTS transcription factor	3.20	5.64	0.57
PGA11	Putative GPI-anchored protein	1.16	2.03	0.57
TPK1	cAMP-dependent protein kinase	2.32	3.86	0.60
GIT1	Glycero phosphodiester transporter	1.30	2.14	0.60
C1_01130W_A	Putative ubiquitin ligase complex component	1.99	3.24	0.61
WOR1	Transcription factor of phenotypic switching	1.32	2.16	0.61
IFE2	Putative alcohol dehydrogenase	1.50	2.41	0.62
SFL2	Transcription factor required for filament	1.79	2.84	0.63
HAP2	Transcription factor of low-iron induction	1.37	2.16	0.63
PBR1	Protein required for biofilm formation	2.51	3.92	0.64
SWE1	Putative protein kinase required for virulence	1.36	2.11	0.64
AHR1	Transcription factor involved in adhesion	1.76	2.62	0.67
HYR1	Hyphal cell wall protein involved in adhesion	6.82	9.87	0.69

biofilms formed by *C. albicans* was observed by SEM. In the absence of progesterone *C. albicans* SC5314 cells formed a multilayer and compact biofilm that covered the entire surface, constituted by a dense network of hyphal forms (Fig. 1D). Differently, in the presence of 2 μM of progesterone, the biofilm consisted of a discontinuous multilayer with a lower number of cells. These observations are consistent with the reduction in the number of cultivable cells (Fig. 1A) and total biomass (Fig. 1B) of *C. albicans* SC5314 biofilms.

3.2. Transcriptional profiling of C. albicans cells present in biofilms formed in the presence or absence of progesterone

To gain further insights into the molecular mechanisms by which progesterone affects the physiology and response of *C. albicans* SC5314 cells, the transcriptomes of biofilms formed in the absence and presence of progesterone were compared (after 24 h of cultivation in RPMI medium at pH 4, either supplemented or not with 2 μM of the hormone). Because a direct comparison of these two datasets could result in the identification of genes responding to stimuli other than just progesterone (e.g. to the acidic pH conditions used) we have compared each set of biofilm cells against the transcriptome of planktonic *C. albicans* SC5314 cells. By doing this we could identify in a first stage the genes changing their expression in the biofilms formed under the acidic conditions and afterwards use this information to pinpoint the progesterone-responsive genes. This setup also allowed us to obtain a portrait of the transcriptome of mature biofilms formed by *C. albicans* SC5314 in acidic conditions, something that, to our knowledge, had not been performed before, since previous studies were performed using biofilms formed in near-neutral pH (García-Sánchez et al., 2004; Murillo et al., 2005; Yeater et al., 2007). Only genes whose transcripts

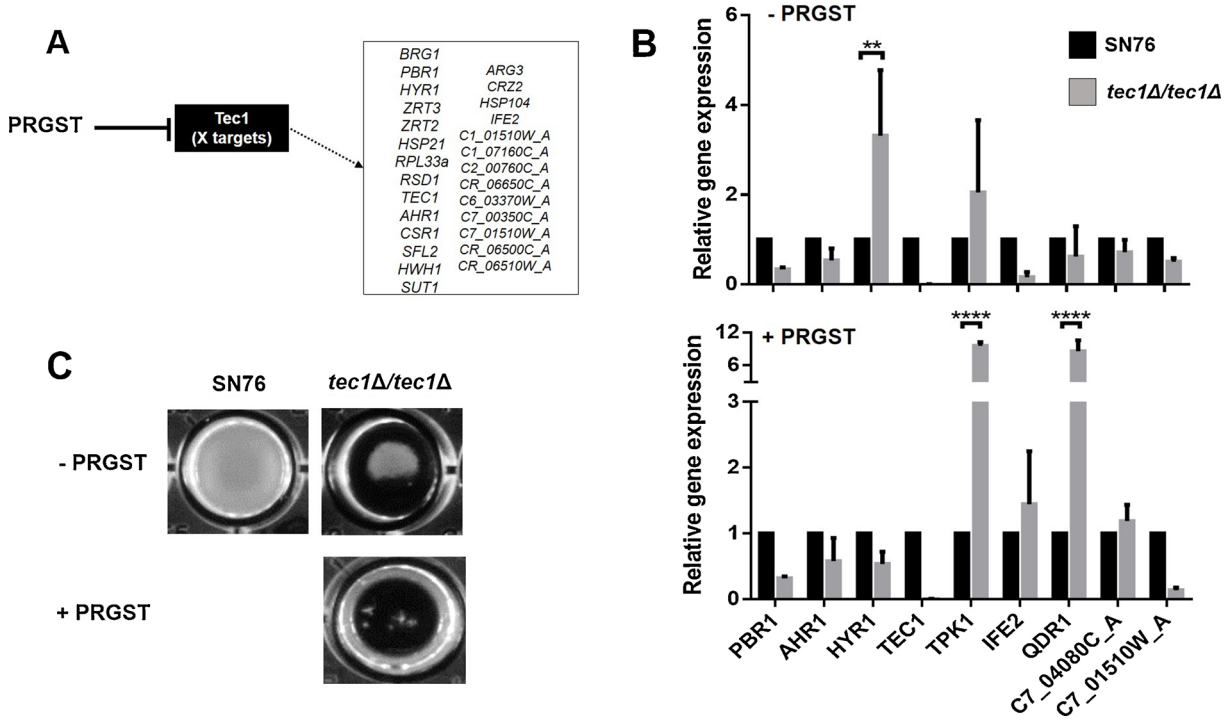

Fig. 5. Tec1-documented targets that were found to have a reduced expression in progesterone-exposed biofilm cells (A and B).The transcriptome-wide analysis carried out led to the identification of a set of genes (fully detailed in supplementary Table S5) that were found to have a reduced expression in progesterone-exposed biofilm cells, in comparison with the level attained in biofilms not exposed to the hormone. This set of genes included Tec1 and also a set of documented targets. **(A)** In the picture are depicted all those documented Tec1 targets that have a biological function related to biofilm formation or whose expression was found to be induced along biofilm formation. **(B)** The effect of Tec1 in up-regulating genes previously shown as relevant for *C. albicans* biofilm formation was tested by real-time PCR, being confirmed the positive effect in the expression of *PBR1*, *AHR1* and *C7_01510w_A*, both in absence of the hormone (-PRGST) and in progesterone-exposed biofilm cells (+PRGST). Consistent with these results, Tec1 was found to be essential for formation of acidic biofilms and this phenotype was aggravated in the presence of progesterone **(C)**. Error bars represent standard deviation. Asterisks represent statistical difference between the results of the mutant and parent strain. (**** p-value ≤0.0001; ** p-value ≤0.01).

increased by more than 2-fold in the biofilm cells (in the presence or absence of progesterone), in comparison the expression levels registered in planktonic cells, were considered. A more detailed analysis on the observed transcriptome-wide alterations of *C. albicans* SC5314 biofilm cells follows.

3.3. Transcriptome-wide alterations of C. albicans biofilm cells under acidic conditions

Transcriptional profiling of *C. albicans* biofilms formed after 24 h of cultivation in acidic RPMI medium (at pH 4) showed a significant (p-value below 0.01) alteration (above or below 2-fold) in the expression of 1013 genes. Specifically, 616 genes were up-regulated in biofilm cells, while 397 genes were down-regulated, in comparison with the transcript levels observed in planktonic cells. A subset of these genes is listed in Table 2 and the full list is available in supplementary Table S2. About 322 of the up-regulated genes were previously shown to be induced during biofilm formation by *C. albicans* (Desai et al., 2013; García-Sánchez et al., 2004; Murillo et al., 2005; Nett et al., 2009; Yeater et al., 2007). However, 294 genes are reported to be up-regulated in biofilm cells for the first time (that is, these genes were not reported to change their expression in other studies that examined transcriptomic responses of *C. albicans* biofilm cells under other experimental conditions) suggesting that they can be responding to the acidic environment (highlighted in grey in Table 2 and supplementary Table S2). Notably, 8 of these newly identified biofilm-responsive genes are essential for biofilm formation (highlighted in bold in Table 2 and supplementary Table S2): *NDT80* and *WOR1*, encoding two transcriptional regulators (Nobile et al., 2012; Yi et al., 2011); *ALS5,* encoding an adhesin (Garcia et al., 2011); the membrane protein *PGA10*; *CSA2*, encoding a cell surface protein involved in iron utilization (Srikantha et al., 2013), and the poorly characterized genes *NPL3*, *C1_00160C_A* and *C7_00490C_A* (Bonhomme et al., 2011). Further studies will be required to confirm whether or not the other newly identified biofilm responsive genes also play a relevant role in the formation of biofilms under acidic conditions.

To get a clearer picture of the alterations occurring in the genomic expression of *C. albicans* biofilm cells under acidic conditions, the genes up and down-regulated were functionally clustered using the MIPS Functional catalogue (Fig. 2). The functional classes enriched (p-value below 0.001) in the dataset of up-regulated genes were “transcription”, “protein synthesis”, “protein with binding function” and “transport”. On the other hand, down-regulated genes were enriched in functions involved in “metabolism” (including enrichment of the subclasses “amino acid”, “nitrogen”, “carbohydrate”, “lipid/fatty acid” and “secondary” metabolisms), “generation of energy”, “protein with binding function”, “transport”, “stress response” and “interaction with the environment” (Fig. 2). In general, the functional clustering of the genes found to be differently expressed in our acidic biofilms is similar to those found in other studies (Desai et al., 2013; García-Sánchez et al., 2004; Murillo et al., 2005; Nett et al., 2009; Yeater et al., 2007), something that could be attributable to the fact that most of the genes that we found differentially expressed in our acidic biofilms have a poorly or even uncharacterized function.

To further understand the transcriptional regulatory network active in the formation of biofilms under acidic conditions, we used the PathoYeastract database (Monteiro et al., 2017)to cluster the up-regulated genes with transcription factors reported to control the process of biofilm formation in *C. albicans* (Ndt80, Efg1, Bcr1 and Tec1) (Nobile et al., 2012). Approximately 33 % of the genes up-regulated in the

acidic biofilms are documented targets of Ndt80 transcription factor, 32 % of Efg1, 16 % of Bcr1, 13 % of Tec1 and 13 % of Brg1 (supplementary Table S3). Fig. 3 shows the target genes of Efg1, Brg1, Bcr1 and Tec1 with differential expression under biofilm-forming conditions, according with the information available on the PathoYeastract database (Monteiro et al., 2017). A significant overlap between the genes regulated by each transcription factor was observed, confirming the complex and intertwined nature of the regulatory network controlling expression of biofilm genes in *C. albicans* (Nobile et al., 2012). The high percentage of documented targets of CaNdt80 suggests that this regulator plays a particularly critical role in the control of expression under these conditions. For 248 up-regulated genes we could not establish an association with Efg1, Ndt80, Bcr1 and Tec1 and we have used the PathoYeastract database again in order to identify what could be the potential regulators of these genes. The results show that 160 of these genes are targets of Sfl1. In fact, when we consider the entire dataset of up-regulated genes Sfl1 is predicted to regulate 279 of these, which surpasses the number of targets attributed to Efg1, Ndt80, Bcr1 or Tec1 (Fig. 3). These two observations suggest that this transcription factor plays a role in formation of biofilms under acidic conditions, a hypothesis that was confirmed when we compared the ability of wild-type and *sfl1*Δ/*sfl1*Δ cells to form biofilms under acidic conditions (Fig. 4A). Recently, Sfl1 was found to be required for formation of microcolonies contributing to maximal adhesion to epithelial cells (McCall et al., 2018). The involvement of Sfl1 in formation of acidic biofilms represents a further insight into the biological function of this regulator.

The high overlap observed between the activated genes in our acidic biofilms documented to be regulated by Sfl1 and Ndt80 prompted us to test whether Sfl1 is a positive regulator of *NDT80* expression. We found that in acidic biofilms formed by the mutant *sfl1*Δ/*sfl1*Δ the expression of *NDT80* is approximately 10 % of the levels in the parental strain (Fig. 4B). Previous studies have also shown that there is functional interaction between Sfl1 and Ndt80 during hyphae formation in *C. albicans* (Znaidi et al., 2013). Sfl1 was also found to be required for maximal expression of *NPL3* (Fig. 4B), which is up-regulated in acidic biofilms, and has been shown to be an important determinant of biofilm formation in *C. albicans* (Bonhomme et al., 2011). Further studies are required to fully characterize the pH-dependent regulation of gene expression by Sfl1 during biofilm formation and, in particular, to identify Sfl1-regulated targets that are essential for biofilm formation. Although Sfl1 has been mostly described as a transcriptional repressor in *C. albicans* (Bauer and Wendland, 2007; McCall et al., 2018), evidences from chromatin immunoprecipitation profiling have shown that it also acts as a positive regulator (Znaidi et al., 2013). Similarly, the ScSfl1 orthologue has also been found to have a dual effect acting both as a positive and a negative regulator (Conlan and Tzamarias, 2001). The molecular mechanisms driving the regulation of the activity of Sfl1 under the different environmental contexts that may control its activity as an activator and/or repressor and how those environmental cues are transduced into that regulatory mechanism remain to be established.

3.4. Transcriptome-wide alterations in C. albicans acidic biofilms in the presence of progesterone

Transcriptome-wide profiling of *C. albicans* biofilms obtained after 24 h of cultivation in RPMI medium (at pH 4) supplemented with 2 μM of progesterone identified a significant (*p*-value below 0.01) alteration (above or below 2-fold) in the expression of 1057 genes (556 up- and 501 down-regulated), compared to the expression that was registered in planktonic cells (supplementary Table S4). A large overlap between this dataset and the dataset of genes differentially expressed in the biofilms formed without progesterone (described above) was observed (supplementary Fig. S2). This indicates that most of the changes observed in the progesterone-exposed biofilms result from the process of biofilm formation itself and not from the direct exposure to the hormone. We have considered as progesterone-responsive genes only those whose level of induction or repression in the presence of the hormone was 40 % higher than the levels registered in the absence of it. Using this criterion, 220 progesterone-responsive genes could be identified, these being afterwards divided in four groups: *i)* genes up-regulated in biofilms formed in the presence or absence of progesterone, but showing a stronger induction in the presence of the hormone (42 genes); *ii)* genes more strongly up-regulated in biofilms formed without progesterone (87 genes); *iii)* genes more strongly down-regulated in biofilms formed without progesterone (12 genes); *iv)* genes more strongly down-regulated in the progesterone-exposed biofilms (79 genes). A subset of these progesterone-responsive genes is shown in Table 3 and the full list is available in supplementary Table S5.

Closer analysis of the set of genes with higher induction in the progesterone-exposed biofilms (clustered in group *i)* revealed a prominent increase in transcription of *CDR1* and *CDR2*, encoding two multidrug resistance transporters of the ABC Superfamily (Table 3). The up-regulation of *CDR1* in response to progesterone in *C. albicans* biofilm cells was further confirmed by qRT-PCR (supplementary Fig. S3). Other studies have also reported up-regulation of *CDR1* transcription upon exposure of planktonic *C. albicans* cells to progesterone and to female serum (Karnani et al., 2004; Larsen et al., 2006). Consistent with this observation, planktonic progesterone-exposed cells are less sensitive to fluconazole than cells cultivated in the absence of the hormone (Banerjee et al., 2007; Larsen et al., 2006). Similarly, we observed that progesterone-exposed biofilm cells were significantly more tolerant to fluconazole than cells of biofilms developed in the absence of the hormone (MIC of 1.5 μg/mL compared with 0.25 μg/mL).

The inhibitory effect of progesterone in biofilm formation may be related to reduced expression of genes required for this process. Expression of 166 progesterone-responsive genes is reduced in biofilm cells grown with progesterone (87 clustered in group *ii* and 79 in group *iv* (supplementary Table S5).These include several key regulators of biofilm formation, including the transcription factors Tec1, Brg1, Ahr1, Wor1, Csr1 and Crz2 (Askew et al., 2011; Finkel et al., 2012; Nobile et al., 2012, 2009; Yi et al., 2011), the heat shock protein Hsp104 (Fiori et al., 2012) and the uncharacterized protein Pbr1 (Sahni et al., 2009), among other genes previously shown to be required for biofilm formation (all highlighted in grey in supplementary Table S5). The progesterone-decreased expression of *TEC1* and *CRZ2* genes in *C. albicans* biofilm cells was further confirmed by qRT-PCR (supplementary Fig. S3).

Several genes involved in the process of biofilm formation that had a reduced expression in the presence of progesterone are documented targets of Tec1 (Fig. 5A) and Brg1, according to the PathoYeastract database (Monteiro et al., 2017). We therefore tested whether these two regulators are required for biofilm formation in the presence of the hormone. Under the conditions that we have used only the deletion of *TEC1* had a significant effect in formation of acidic biofilms, this phenotype being further aggravated in the presence of progesterone (Fig. 5C and results not shown for *BRG1* deletion). Consistently, Tec1 was required for maximal expression of *IFE2*, *PBR1* and *C7_01510W_A*, either in the presence or absence of progesterone (Fig. 5B). Tec1 is also required for maximal expression of *HYR1* in progesterone-exposed biofilm cells. However, in the absence of the hormone expression of *HYR1* is increased in the *tec1*Δ/*tec1*Δ mutant strain (Fig. 5B). This observation is a clear reflection of the complex nature of the regulatory networks governing biofilm formation in *C. albicans*, which can be greatly shaped by the environmental conditions.

4. Conclusions

Our study examined, for the first time, the alterations occurring in the genomic expression of *C. albicans* during biofilm formation in acidic conditions (RPMI at pH 4), which could potentially lead to the identification of novel players required for maximal biofilm formation in the acidic vaginal tract. Indeed, we identified 286 genes whose

transcription was changed during biofilm formation that have not previously been associated with this process. These genes and their regulators represent thus an interesting cohort to search for new players involved in biofilm formation in the acidic vaginal tract. Using this rationale, we showed that Sfl1 is essential for maximal biofilm formation and that is also likely to play a significant role in regulation of gene expression in acidic-biofilm cells. In particular, Sfl1 regulates of Ndt80, a major regulator of biofilm development. The impact of progesterone exposure in biofilm formation and in genomic expression of biofilm-forming *C. albicans* cells was also examined in our work for the first time. The reduced ability to form biofilms observed upon exposure to progesterone was consistent with the reduced transcription of several key genes required for maximal biofilm formation, including the transcriptional regulator Tec1 and several of its target genes. Progesterone exposure was also found to significantly decrease the susceptibility of biofilm cells to fluconazole, which is attributable to a reduction in expression of the drug-efflux pump *CDR1*. Considering that the vaginal tract is one of the ma-in driveways for the development of *C. albicans* infections, the identification of genes that may determine the ability of this yeast to survive in specific vaginal conditions as the acidic pH or progesterone presence may contribute to the disclosure of new targets to treat VVC infections.

Funding

This work was supported by the Portuguese Foundation for Science and Technology (FCT) under the scope of the strategic funding of UID/BIO/04469/2019 unit and BioTecNorte operation (NORTE-01-0145-FEDER-000004) funded by the European Regional Development Fund under the scope of Norte2020 – Programa Operacional Regional do Norte. Funding received by iBB–Institute for Bioengineering and Biosciences from FCT (UID/BIO/04565/2013), from Programa Operacional Regional de Lisboa 2020 Project No. 007317 is also acknowledged. Support from FCT to NAP through the doctoral program Applied and Environmental Microbiology (PD/BD/143026/2018) is also acknowledged. B. Gonçalves is recipient of a PhD grant supported by FCT (SFRH/BD/111645/2015). G. Butler was supported by Science Foundation Ireland (12/IA/1343).

Declarations of Competing Interest

None.

Acknowledgments

The authors thank Drs. Christophe d'Enfert, Karl Kuchler, Sophie Bachellier-Bassi and Sabrina Jenull for kindly providing mutant strains.

Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.ijmm.2020.151414.

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