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Interaction between *Candida albicans* and *Pseudomonas aeruginosa*

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By

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For my daughter, Arlene (Arli), and my family

...

Thank you for everything, especially for your patience

...

Declaration

I, the undersigned Nina Konstantinidou, declare that I have not obtained a degree from the University College Cork, National University of Ireland, Cork, or elsewhere on the basis of this Ph.D. Thesis. The results presented in this thesis were derived from the experiments undertaken by myself in the laboratories of the University College Cork.

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Νίνα Κωνσταντινίδου

Publications

Peer reviewed publication

The results from the Chapters 2 and 3 were published in FEMS Yeast Research:

- Konstantinidou N & Morrissey JP (2015) Co-occurrence of filamentation defects and impaired biofilms in *Candida albicans* protein kinase mutants. FEMS Yeast Res 15: 1–10. pii fov092 doi: 10.1093/femsyr/fov092.

Public access database

A TFbsST (**T**ranscription **F**actor **b**inding **s**ite **S**earch **T**ool) database was published online under the following link:

- http://bioinfo.ucc.ie/TFbsST/

Chapter 4 is in preparation for submission in the Database Issue of Nucleic Acid Research (Oxford Journals).

General scientific communication

Parts of the Chapter 1 were published in the following articles:

- Konstantinidou N (2014) Decoding bug chatter to fight infections. The Boolean 4: 51-55.
- Konstantinidou N (2014) Membership Q & A. Microbiology Today Magazine 41: 82-83.

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List of Abbreviations

Abbreviation	Term
%	Percentage
3O-C_{12}-HSL	*N*-(3-oxododecanoyl)-L-Homoserine Lactone
A_{600}	Absorbency at 600 nm wave length λ (Lambda)
AIDS	Acquired Immune Deficiency Syndrome
Arg	Arginine
bp	Base pair
C_4-HSL	*N*-butyryl-L-Homoserine Lactone
cAMP	Cyclic Adenosine Monophosphate
CF	Cystic Fibrosis
CGD	*Candida* Genome Database
CDR	*Candida* Drug Resistance genes
DBD	DNA-Binding Domain
DBD database	DNA-Binding Domain database of predicted transcription factors
dH_2O	Distilled water
DNA	Deoxyribonucleic acid
DRE	Drug Responsive Element
ECM	Extracellular Matrix
EDTA	Ethylene-Diamine-Tetraacetic Acid
EMSA	Electrophoretic Mobility Shift Assay
g	Gram
GO	Gene Ontology
h	Hour
HEA	Higher Education Authority
HHQ	2-Heptyl-4-Quinolone (PQS precursor)
His	Histidine
HSL	*N*-acyl-L-Homoserine Lactone
HIV	Human Immunodeficiency Virus
HTML5	Hyper Text Markup Language 5
ID	Identifier
KEGG	Kyoto Encyclopaedia of Genes and Genomes
LB	Luria-Bertani or Lysogeny Broth
Leu	Leucine
LHS	Left Hand-Side
LSM	Laser Scanning Microscope
MAPK	Mitogen-Activated Protein Kinase
Mb	Mega base
MDR	Multidrug Resistance genes
Min	Minute
mL	Millilitre
mySQL	My Structured Query Language
n	Number
^oC	Degree Celsius
PCR	Polymerase Chain Reaction
pH	Power of Hydrogen
PK	Protein Kinase
PQS	*Pseudomonas* Quinolone Signal
QS	Quorum Sensing
RAS/cAMP/PKA	Rat Sarcoma/cyclic Adenosine Monophosphate/Protein Kinase A

RNA	Ribonucleic acid
rpm	Revolutions or Rotation Per Minute
S	Supplementary
s/n	Supernatant
sec	Second
STRING	Search Tool for the Retrieval of Interacting Genes/Proteins
TAD	Trans-Activating Domain
TF	Transcription Factor
TFbsST	Transcription Factor binding site Search Tool
Tm	Melting temperature
Tn7-UAU1	Tn7 unit Transposon (transposable element) *-ura3Δ3'-ARG4-ura3Δ5*
Ura	Uracil
v	Version
XTT	2,3-bis-(2-methoxy-4-nitro-5-sulfophenyl)-2H-tetrazolium-5-carboxanilide
YNB	Yeast Nitrogen Base
YNBNP	Yeast Nitrogen Base *N*-acetyl-D-glucosamine Phosphate
YPD	Yeast extract Peptone Dextrose (rich yeast medium)
WBC	White Blood Cells
Δ (Delta)	Deletion (when used before a gene name)
µg	µicrograms
µL	µicrolitre
µm	µicrometer

Abstract

Fungal pathogen *Candida albicans* causes serious nosocomial infections in patients, in part, due to formation of drug-resistant biofilms. Protein kinases (PK) and transcription factors (TF) mediate signal transduction and transcription of proteins involved in biofilm development. To discover biofilm-related PKs, a collection of 63 *C. albicans* PK mutants was screened twice independently with microtiter plate-based biofilm assay (XTT). Thirty-eight (60%) mutants showed different degrees of biofilm impairment with the poor biofilm formers additionally possessing filamentation defects. Most of these genes were already known to encode proteins associated with *Candida* morphology and biofilms but *VPS15, PKH3, PGA43, IME2* and *CEX1*, were firstly associated with both processes in this study.

Previous studies of Holcombe *et al*. (2010) had shown that bacterial pathogen, *Pseudomonas aeruginosa* can impair *C. albicans* filamentation and biofilm development. To investigate their interaction, the good biofilm former PK mutants of *C. albicans* were assessed for their response to *P. aeruginosa* supernatants derived from two strains, wild-type PAO1 and homoserine lactone (HSL)-free mutant ΔQS, without finding any non-responsive mutants. This suggested that none of the PKs in this study was implicated in *Candida-Pseudomonas* signaling.

To screen promoter sequences for overrepresented TFs across *C. albicans* gene sets significantly up/downregulated in presence of bacterial supernatants from Holcombe *et al*. (2010) study, TFbsST database was created online. The TFbsST database integrates experimentally verified TFs of *Candida* to analyse promoter sequences for TF binding sites. *In silico* studies predicted that Efg1p was overrepresented in *C. albicans* and *C. parapsilosis RBT* family genes.

Summary

C. albicans is a serious human pathogen partially due to its drug resistant biofilm development that depends on the environmental signals sensed by the cell receptors. The PKs and TFs regulate signal transduction and gene transcription process respectively.

The primary aim of this research was to discover the most important PKs for *C. albicans* biofilm development. Indeed, we identified 38 PK mutants with different degrees of biofilm impairment but some of them were already linked with biofilms. Nearly half of these mutants were classified as 'Poor' biofilm formers possessing additional filamentation defects. The novel findings included 5 genes, *VPS15, PKH3, PGA43, IME2* and *CEX1*, not previously associated with either filamentation or biofilm formation. All these 5 genes seem to participate in processes that are important for biofilm formation, validating our approach. For example, even though *VPS15* encodes a kinase involved in vacuolar protein sorting, other members of the Vps protein family, Vps1p and Vps34p, were reported to significantly contribute in *Candida* biofilm formation. Vps15p and Vps34p form a complex on the vacuole/golgi membrane indicating that a proper fungal cell development requires correct protein sorting mechanisms. *PKH3* encodes a kinase that may be involved in Protein Kinase C (PKC) activity, which is important for drug resistance of *C. albicans*. Additionally, the *pkh3* mutant is reported to display significantly decreased cell-substrate adherence, what prevents biofilm formation. Interestingly, Pkh3p was clustered with the vacuolar proteins Vps15p and Vps34p in the network, probably indicating that the defect in this mutant is also related to vacuole protein sorting mechanisms. *PGA43* encodes a Glycosyl-Phosphatidyl-Inositol (GPI)-anchored protein with unknown function but other Gpi family proteins, Pga59p and Pga62p, are known to be required for cell wall integrity, which is mandatory for filamentation. Involvement of *C. albicans* Ime2p in morphological development is not surprising since its *Saccharomyces cerevisiae* orthologue is essential for pseudohyphal growth independent of the MAPK filamentation cascade. The function of *C. albicans* Cex1p is unknown but in *S. cerevisiae* it is exported from the nucleus via tRNA binding.

Apart from vacuolar process, our data showed that the PKA pathway, ribosome biogenesis and some aspects of the cell cycle are also required for efficient filamentation and biofilm development. For example, mutants that were disrupted in *C. albicans* PKA genes, *BCY1*, *TPK1* and *TPK2*, and the ribosome biogenesis genes, *CKA2, CKB1* and *CKB2*, exhibited impaired biofilms under our assay conditions. Bcy1p is a regulatory subunit of PKA, which is

prominent for *C. albicans* morphogenesis and, Tpk1p and Tpk2p are well-documented to be involved in filamentation. The ribosome biogenesis genes were also reported to display elevated transcription in biofilm forming cells. Upon these findings, it was interesting to see which proteins were not absolutely required for biofilm formation. Surprisingly, none of the individual PKs in MAPK (Mitogen-Activated Protein Kinase) cascades was essential, indicating that MAPK mutants are compensated by parallel MAPK pathways in the cell.

The second goal of this project was to investigate the interaction between *C. albicans* and *P. aeruginosa*. Previous studies had shown that *Pseudomonas* supernatants could impair both the yeast-hyphal transition and biofilm development in *Candida*. Thus, the 'Good' biofilm former mutants of *C. albicans* were assessed for their response to *P. aeruginosa* supernatants without finding any non-responsive mutants . This result suggested that none of the PKs in this study was implicated in *Candida* signal transduction response to *Pseudomonas* signals. However, the comprehensive analysis of the mutants in presence of supernatants derived from *N*-acyl-L-Homoserine Lactone (HSL)-positive and HSL-negative strains of *P. aeruginosa* showed 2 distinct effects on *Candida*: HSL-dependent impairment of morphology and HSL-independent impairment of biofilms.

To further investigate the TF regulation of *Candida* genes that were altered in presence of *P. aeruginosa* secreted chemicals, we created a TFbsST database (http://bioinfo.ucc.ie/TFbsST/). *Candida* TF library with experimentally validated motifs and Python scripts were integrated to develop a user-friendly application for the analysis of gene promoter regions. The TFbsST database includes TFs of *C. albicans* and closely related *Candida* species such as *C. parapsilosis, C. dubliniensis* as well as evolutionary distinct *C. glabrata*. Initially, we annotated the differentially expressed genes of *C. albicans* that were up/downregulated in response to *P. aeruginosa* supernatants and shortlisted the genes coding for cell wall/surface proteins including members of *RBT* and *ALS* families. Using TFbsST database, we showed that several members of both gene families possessed Efg1p binding sites in their promoters enhancing the importance of Efg1p in the yeast to hyphae switch. The presence of Efg1p binding motifs in *C. parapsilosis RBT* family gene promoters further supported its regulatory role across the *Candida* spices.

Conclusively, our approach, bioinformatics tools and data generated from this study seed into the existing models of *C. albicans* and increase our understanding of its cellular mechanisms.

Chapter 1

General Introduction

Parts of this chapter were published in The Boolean (Konstantinidou N (2014) Decoding bug chatter to fight infections. Boolean 4: 51-55) and *Microbiology Today* (Konstantinidou N (2014) Membership Q & A. MT magazine 41: 82-83).

1. General introduction

1.1 *Candida albicans* virulence

C. albicans lives in healthy human host as a commensal colonising the mucosal microflora of urogenital and gastrointestinal tracts as well as oral cavity. However, *C. albicans* becomes pathogen after the overgrowth of the communities that can cause infectious diseases by bypassing the hosts defence system. *C. albicans* can overcome the macrophage innate immunity barrier with the metabolic changes mediated by the members of the Ato protein family (Danhof and Lorenz 2015) and the excretion of farnesol that stimulates macrophage chemokinesis (Hargarten *et al*. 2015). *Candida* infections range from life-threatening invasive candidiasis (candidaemia) to superficial mucosal infections known as 'thrush'. Other conditions caused by *Candida* include oral, mucosal and dermatological candidiasis; lung, hepatic (liver) and renal (kidney) abscess; pyelonephritis, vulvovaginitis and candiduria; as well as osteomyelitis (bone marrow), nail and eye infections (reviewed by Gulati and Nobile 2016) (Figure 1 and Table 1).

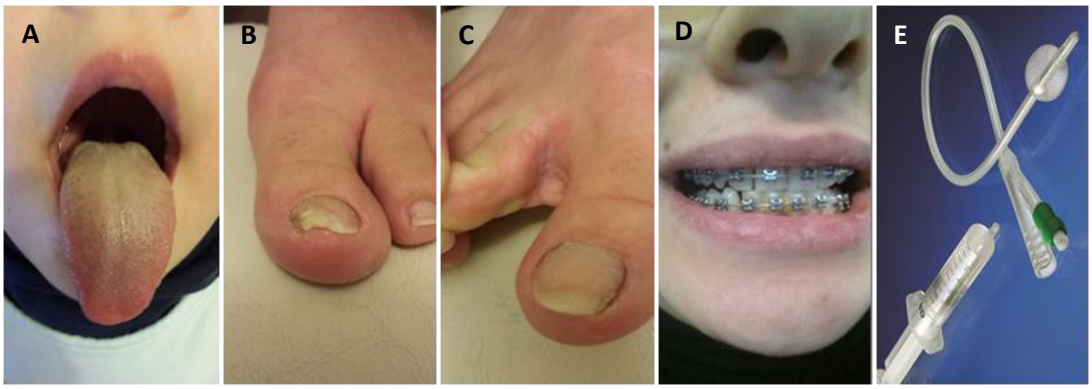

Figure 1| **Some examples of yeast infection. A**| Oral infection in children, **B**| Nail infection, **C**| Skin infection, **D**| Plaque due to dental appliances and **E**| Biofilms on catheters causing bloodstream infections. The images were taken in this study with the patients' consent under the confidentiality policy of the Irish Health Care Board.

The main groups of patients vulnerable to *Candida* infections include immunocompromised populations, due to cancer treatment (with chemotherapy), organ transplant (with immunosuppressants) and HIV (with immunodeficiency virus). *Candida* profits from the dysfunction of the immune system to dominate and infect the patients with immune

disorders. Another group involves the cystic fibrosis (CF) patients with depleted defence bacteria due to the widespread use of antibiotics that promotes growth of *C. albicans* communities. Along with the pathogens (usually *Pseudomonas aeruginosa*), the antibiotics also eradicate the commensal bacteria that activate hypoxia-inducible factor-1α (HIF-1α) and the antimicrobial peptide LL-37 of a host preventing host response to *C. albicans* infections (Fan *et al*. 2015). Additionally, enhanced growth of *Candida* colonies was observed in the cortisone administered patient groups (Seligmann 1953).

C. albicans is the 4th common pathogen that causes infections in nosocomial patients and accounts for 30% mortality rate mainly because of invasive bloodstream infections. *C. albicans* pathogenesis is enhanced with its virulence factors such as adhesins (biomolecules that facilitate host recognition and cell adhearance), aspartyl proteases and phospholipases (secreted molecules), morphogenesis (reversible transition forms between unicellular yeast and filamentous hyphae) and biofilms (complex community structures protected with extracellular matrix). The bloodstream infections are seeded by the *C. albicans* biofilms that are developed on implanted medical devices and catheters. Drug resistant biofilms covered by the extracellular matrix (ECM), protect *Candida* communities from antifungals and antibodies of the immune system making *Candida* infections practically untreatable.

Table 1| *Candida* infection manifestations at different body sites.

Organs	Superficial	Bloodstream
Eye infection	Dermatological candidiasis	Candidaemia
Cerebral candidiasis	Mucosal candidiasis	Osteomyelitis
Lung abscess	Oral candidiasis	Medical implants
Hepatic abscess (liver)	Nail infection	Catheters
Renal abscess (kidney)		
Pyelonephritis (kidney)		
Vulvovaginitis (vagina)		
Candiduria (bladder)		

1.2 Drug resistance strategies of *Candida albicans*

C. albicans biofilms are widely investigated due to their connection with drug tolerance and high mortality rates. In addition to the switching ability, the virulence of *Candida* is increased by its extracellular matrix (ECM) that contains proteins, polysaccharides and extracellular DNA. ECM covers the biofilms and prevents the action of the antifungals. *Candida* drug resistance genes (*CDR* and *MDR*) that are upregulated during biofilm

formation, encode for 2 types of efflux pumps, ABC transporters (*CDR*) and MFS (major) facilitators (*MDR*) further increasing drug tolerance of *Candida* (Ramage *et al*. 2002). *C. albicans* communities in the biofilms (only) also contain persister cells, variants of normal cells that are usually in a dormant state, surviving the high doses of antifungals (LaFleur *et al*. 2006; Lewis 2012). Due to the latter, the commercially available antifungals (azoles, polyenes and echinocandins) are often ineffective against *Candida* biofilms. Azoles inhibit lanosterol, polyenes target ergosterol of cell membrane and weaken it, and echinocandins inhibit the synthesis of glucans in the fungal cell wall. Ineffectiveness of these drugs directs research towards alternative therapeutic strategies. These strategies were reviewed by Nett (2014) and include targeting extracellular matrix (ECM) and quorum sensing (QS) (Nett 2014). The recent insights in the field of biofilms and drug resistance have also highlighted the role of combination therapy that includes the use of the antifungals and the natural compounds derived from the plants (e.g. menthol, nerol), fungi (e.g. penicillin) and bacteria (e.g. *Pseudomonas* phenazines and QS molecules) (Kerr *et al*. 1999; Hogan and Kolter 2002; Hogan *et al*. 2004; McAlester *et al*. 2008; Deveau and Hogan 2011). This approach reduces the probability for resistance development which is the main issue for the fungal infection treatments.

1.3 *Candida albicans* morphology and morphogenesis

The human pathogen, *C. albicans*, is a polymorphic fungus with a complex life cycle. In order to adapt to new environments it develops a full repertoire of distinct morphological forms including budding yeast (blastospore), intermediate pseudohyphae (Sudbery *et al*. 2004), filamentous hyphae, mycelium with secondary blastospores and biofilms with extracellular matrix (ECM) (recently reviewed in detail by Nobile and Johnson 2015). The ploidy plasticity of *C. albicans* allows rapid adaptation to the stressful conditions (Berman 2016). Different cell types vary in terms of polarization degree, septum position and nucleus movement. However, yeast, pseudohyphae and hyphae possess a single nucleus in each cell before mitosis. Uhl *et al*. (2003) identified 146 genes that are involved in switch between yeast and filamentous growth. The morphological switch between these forms represents a crucial factor for *C. albicans* virulence (Calderone and Fonzi 2001) (Figure 2).

Apart from yeast to hyphae switch, the white round yeast cells can also be transformed into the elongated opaque cells reversibly and proliferate by mating projection (Slutsky *et al*. 1987; Rikkerink *et al*. 1988; Magee and Magee 1997; Molero *et al*. 1998). Ssn6p plays an important role in white-opaque switching (Hernday *et al*. 2016). *C. albicans* gray phenotype

was recently described by Tao *et al*. 2014. Additionally, *C. albicans* has the ability to form chlamydospores with thicker cell wall in response to the nutrient or environmental stress (Fabry *et al*. 2003). Chlamydospores are larger than blastospores and possess thicker cell wall. In order to develop the chlamydospores, *C. albicans* mainly requires 6 genes (*ISW2, MDS3, RIM13, RIM101, SCH9* and *SUV3*) (Nobile *et al*. 2003) and a MAPK Hog1 (Alonso-Monge *et al*. 2003). However, *C. albicans* biofilms represent the most intriguing topic that has received enhanced scientific focus because they are responsible for virulence and drug resistance that lead to increased morbidity and enormous economic expenditure (Brajtburg *et al*. 1981; Lamfon *et al*. 2004).

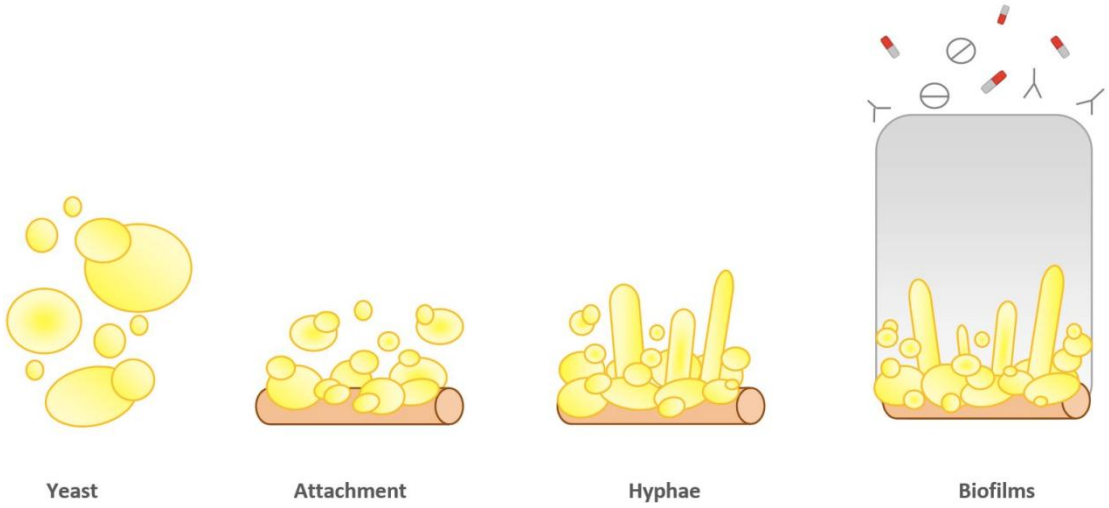

Figure 2| Schematic of *C. albicans* biofilm development. To develop drug resistant biofilms, yeast cells of *C. albicans* attach on the surface and adhere to the neighbouring cells. After filamentous growth of hyphae the matrix, that covers and protects the cells from the antifungals and antibodies, is produced extracellularly. Adapted from Fox and Nobile (2012). Konstantinidou (2014).

Morphogenesis of *C. albicans* is initiated by external signals that are sensed by the cell receptors. The basic environmental factors which are known to induce fungal filamentaion *in vitro* include body temperature (37^{o}C), neutral pH (7.0) and low cellular density (~10^{6} blastospores/ml) as well as presence of compounds such as blood serum, proline (amino acid), glucose (carbon source) and *N*-acetyl-glucosamine (Mattia *et al*. 1982; reviewed by Whiteway and Oberholzer 2004). In order to control its morphogenesis *C. albicans* integrates the environmental stimuli with different signalling pathways. For example, cellular morphogenesis is regulated by calcium signalling pathways (Sanglard *et al*. 2003; Xu *et al*. 2015), yeast to hyphae transition is governed by Ras/cAMP/PKA pathways (reviewed

by Hogan and Sundstrom 2009), hyphal development is coordinated by adenylyl cyclase (cAMP) pathways (Rocha *et al*. 2001) and pseudohyphal induction is orchestrated by MAPK pathways (reviewed by Srinivasa *et al*. 2012). These pathways involve some key transcriptional factors such as Efg1p, Tup1p, Ssn6p, Nrg1p, Brg1p and Cph1p that play an important role in the signal transduction. For instance, inactivation of the transcription factors Cph1p (MAPK pathway) or Efg1p (Ras/cAMP/PKA pathway) can inhibit hyphal growth (Lo *et al*. 1997). These transcription factors collaborate with the histone deacetylases for the morphological transition of *C. albicans* (reviewed by Kim *et al*. 2015). Deacetylases are the enzymes that can remove acetyl groups from the amino acids of a histone allowing DNA to wrap tighter around the histone.

1.4 Biology of yeast, pseudohyphae and hyphae

Early publications have broadly noted the pleiotropic pathogenicity of *C. albicans*. *Candida* causes denture stomatitis (Lilienthal 1955), asthma (Huguenin-Dumittan and Girard 1972), vaginitis (Banner 1974), endocarditis (Calderone *et al*. 1978), septicaemia (Rosin 1974) and infects burn wounds (Albano and Schmitt 1973). The different morphological forms of *C. albicans* induce distinct T helper (Th) cell responses during adaptive immunity providing tissue-specific protection (Kashem *et al*. 2015). The Th17 and Th1 cell responses provide protection against cutaneous and systemic infections respectively. For instance, the yeast form of *Candida* drives Th17 cell response while a filamentous *Candida* induces Th1 cell response (Kashem *et al*. 2015). *C. albicans* morphological forms including yeast, pseudohyphae and hyphae, play a determinant role in fungal virulence (Figure 3).

1.4.1 Yeast

The simplest form of *C. albicans* is a round unicellular yeast (blastospore) formed vegetatively (asexual cell division) via the budding process. Budding cell selection is a temperature dependent phenomenon. Under propitious conditions septin rings signal nuclei to divide across the mother bud neck by asymmetric budding (Sudbery 2001). Fungal isotropic growth is characterised by actin polarization. After cytokinesis, the smaller daughter cell disassociates from the mother cell and enters the next cell cycle once it reaches the threshold size (Figure 3A). A sexual mating and a white-opaque switching can be regulated through the pH of the environment (Sun *et al*. 2015).

1.4.2 Pseudohyphae

The defining characteristic of *C. albicans* pseudohyphal cells is their ellipsoid shape. They bud in an unipolar manner although septin rings appear before the budding process like in

the yeast cells (Sudbery 2001). However, pseudohyphal cells stay in G2 phase longer than the yeast cells. During the polarized pseudohyphal growth, each cell remains attached to another but they are separated by the septa, forming the pseudofilamentous pattern. The new cells enter the next cell cycle in a more synchronized manner than the yeast cells. The elongated pseudohyphal cells form chains and rough colonies after cytokinesis (Figure 3B). The pseudohyphae-associated genes are expressed at low levels and represent a small subset of the hypha-related genes (Carlisle and Kadosh 2013).

1.4.3 Hyphae

C. albicans filamentous growth is regulated independently from its cell cycle (Hazan *et al*. 2002). The yeast-hyphal transition is driven by the extended duration of filament-specific gene expression (Carlisle and Kadosh 2013). *C. albicans* true hyphal cells possess considerable elongation, parallel walls and extreme degrees of polarity (Figure 3C). Polarized growth mode implicates polarisome elements as well as septins, tag/bud site components, Cdc42 module (cell division control protein) and actin-myosin system (Whiteway and Oberholzer 2004). The hyphal cell nucleus divides in the elongated germ tube. Expansion of the filaments is initiated with the asynchronous cell cycle since solely the apical cells start dividing whereas the subapical cells remain in the primary cell phase G1 (Barelle *et al*. 2003). Transcriptional regulator Ash1, which controls filamentous growth, is widely associated with the asymmetric cell division as it is found in the nucleus of apical but not of subapical cells (Inglis and Johnson 2002). Asymmetric vacuolar inheritance also appears to be vital in the hyphae formation (Barelle *et al*. 2003). The true filaments develop via germ tube elongation process and filamentous cells display no distinct constrictions (Berman and Sudbery 2002). Hyphal formation is correlated with the bioactivity of small GTPases (Cdc42/Cdc24 (Ushinsky *et al*. 2002; Bassilana *et al*. 2003)), myosins (molecular motors Myo3/5 (Oberholzer *et al*. 2002)) and PKs (Hsl1p (Umeyama *et al*. 2005) and Cek1p (Csank *et al*. 1998)) that are likely to interact with the actin network.

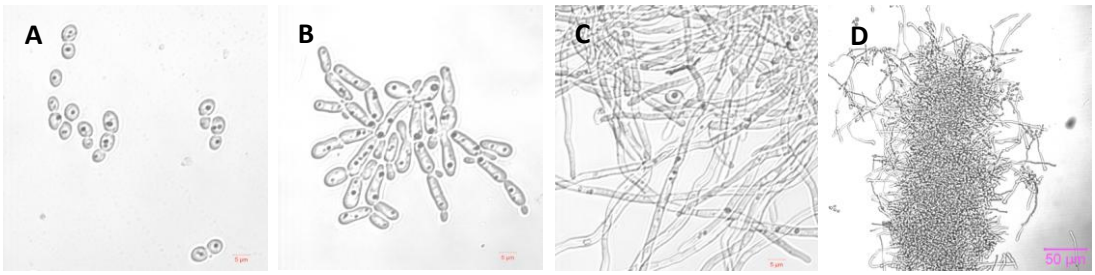

Figure 3| Morphological forms of the wild-type *C. albicans* DAY286 grown in filament-inducing medium at 37°C. A| Yeast (0 h), **B**| Pseudohyphae (6 h), **C**| True hyphae (6 h), **D**| Biofilm (10 h). The images were prepared in this study via a Zeiss Laser Scanning Microscope (LSM) 510 using Zen 2008 software with EX405 BP, DICII and Channel D at a magnification x 400. Scale bars in the first three panels and the last panel represent 5 µm and 50 µm respectively.

In order to form true hyphae, *C. albicans* requires physiological biosynthesis of the cell wall. Due to the latter, mutations in the cell wall synthesis regulatory genes, *SSK1* (Chauhan *et al*. 2003) and *PIR32* (Bahnan *et al*. 2012), may demonstrate various impacts on the hyphal growth. The ability of *C. albicans* to switch from yeast to hyphae is highly connected to the fungal pathogenic lifestyle since nonhyphal mutants are avirulent (Lo *et al*. 1997). For instance, Guhad *et al*. (1998) showed that MAPK (Cek1) defective *C. albicans* mutant was avirulent since it established abnormalities in yeast to hyphal transition (Guhad *et al*. 1998). Usually, yeast or hyphal locked mutants of *C. albicans* are attenuatd in virulence. Recently, *C. albicans* virulence was also linked with mitochondrial activity through the Ras1p regulation (Grahl *et al*. 2015). However, the reverse hyphae-yeast transition of *Candida* involves downregulation of the hyphal-specific genes and differential expression of protein synthesis-related genes (Carlisle and Kadosh 2013).

1.5 *Candida albicans* biofilms

Candida biofilms have received increased scientific interest because they enhance *C. albicans* pathogenicity and drug tolerance. Lately, biofilm development has been the subject of a myriad interesting studies (Chandra *et al*. 2001; García-Sánchez *et al*. 2004; López-Ribot 2005; Finkel and Mitchell 2011; Fox and Nobile 2012; Inglis *et al*. 2013). *In vivo*, biofilms comprise polymicrobial communities where fungi and anaerobic bacteria interact (Fox *et al*. 2014). The hyphae formation is crucial for fungal biofilm development, virulence and drug resistance. Also, hyphal branching ability determines the dynamics of

the biofilm morphology. *In vitro*, the biofilm development demonstrates 3 distinct phases (Chandra *et al*. 2001) (Figure 3):

1) Early stage includes yeast cell attachment to the substrate and adhesion to the neighbouring cells.

2) Intermediate phase involves pseudohyphal and hyphal filamentous growth and branching.

3) Maturation consists from 2 distinct processes, excretion of the extracellular matrix (ECM) and blastospore dispersal. The blastospores are asexual yeast cells that facilitate colonisation of new environments. Blastospore dispersal, biofilm formation and drug resistance are mediated by the histone deacetylase complex (Set3p, Hos2p, Snt1p and Sif2p) (Nobile *et al*. 2014). The ECM of the biofilms is composed from proteins, polysaccharides such as glucose and mannose, lipids and nucleic acid, and contributes to fungal drug resistance (Chandra *et al*. 2001; Fox and Nobile 2012).

Biofilm formation is a complex phenomenon which is orchestrated by more than 1000 proteins that are governed by 9 master regulators, Ndt80p, Efg1p, Brg1p, Bcr1p, Rob1p, Tec1p, Flo8p, Gal4p and Rfx2p (Nobile *et al*. 2012; Fox *et al*. 2015). The biofilm development pathways also include negative regulators like Tup1p and Zap1p which are hyphal (Braun and Johnson 1997; Kebaara *et al*. 2008) and biofilm matrix repressors respectively (Nobile *et al*. 2009).

Table 2|Chemicals that impair biofilm development in *Candida*.

Plant	Bacterium	Human	Other
Terpenoids	Phenazines	Antibodies	Silver nanoparticles
Essential oils	HSLs	Hormones	Povidone-iodine
Purpurin stain	Quinolones	Mucins	Photodynamic therapy
Ethanol	Probiotic products	Milk oligosaccharides	
Propolis			

Despite complexity, *Candida* biofilm development and hyphal induction can be impaired by several plant terpenoids (natural organic chemicals) such as farnesol, linalool, nerol, menthol, carvone and α-thujone (Raut *et al*. 2013); essential oils derived from coriander (Freires *et al*. 2015), thyme (Bogavac *et al*. 2015), pepper (Curvelo *et al*. 2014), tea (de

Campos Rasteiro *et al*. 2014), cinnamon (Pires *et al*. 2011), lemon (Oliveira *et al*. 2014) and carrot (Alves-Silva *et al*. 2016); as well as with propolis (Freires *et al*. 2016). The biofilms are additionally affected by exogenous human hormones including progesterone and oestradiol (Kinsman *et al*. 1988; Zhao *et al*. 1995), the mucins covering the epithelial cells (Kavanaugh *et al*. 2014) and the oligosaccharides contained in the human milk (Gonia *et al*. 2015). Also, biofilms are modulated by application of purpurin (Tsang *et al*. 2012), ethanol (Peters *et al*. 2013) and photodynamic treatment (Sousa *et al*. 2016). Photodynamic therapy was also effective against *Candida* in the murine model *in vivo* (Fabio *et al*. 2016). Recently, the use of povidone-iodine ointment (Hoekstra *et al*. 2016) and silver nanoparticals (drug delivery system) were also proposed as one of the effective strategies for the treatment of *C. albicans* infections (Qasim *et al*. 2015; Wang and Xie 2015). The probiotic supplements containing *Bacillus subtilis* were found to be effective against oral infections caused by *Candida* species (Zhao *et al*. 2016). Apart from these factors, *C. albicans* morphology and biofilm formation is also influenced by some Gram-negative bacteria including *P. aeruginosa* (Peleg *et al*. 2010; Holcombe *et al*. 2011). *P. aeruginosa* phenazines (parent substance of stains/dyes including safranin), pyocyanin, phenazine methosulfate and phenazine-1-carboxylate, affect *C. albicans* biofilm development and metabolism (Gibson *et al*. 2009; Morales *et al*. 2013) (Table 2).

1.6 Interaction between *Candida albicans* and *Pseudomonas aeruginosa*

In clinical settings, the majority of the infections are polymicrobial. Multiple pathogens including yeast, bacteria and viruses, can colonize and infect the same niche. For example, *C. albicans* is often coisolated with an opportunistic human pathogen bacterium *P. aeruginosa*. *C. albicans* and *P. aeruginosa* infections are difficult to treat since both can form biofilms resisting the antifungal and antibiotic treatments respectively (Kojic and Darouiche 2004). *P. aeruginosa* is one of the best studied bacterium found in the plethora of niches due to its extremely adaptable abilities. *P. aeruginosa* can colonise kidneys and urinary as well as gastrointestinal tract of susceptible individuals causing inflammation and sepsis. Additionally, *P. aeruginosa* is the main cause of morbidity in populations with cystic fibrosis (CF) (Govan and Deretic 1996; Chambers *et al*. 2005; Leclair and Hogan 2010) and AIDS (Mendelson *et al*. 1994). Authors of independent studies have established the importance of quorum sensing (QS) system for *P. aeruginosa* fitness (Heurlier *et al*. 2006), virulence (Smith and Iglewski 2003) and inter-kingdom signalling (Shiner *et al*. 2005).

A wide variety of investigations suggest that *C. albicans* and *P. aeruginosa* can coexist and interact in both natural and clinical settings (Hogan and Kolter 2002; Nseir *et al*. 2007; Gibson *et al*. 2009). *C. albicans* and *P. aeruginosa* were coisolated from serious burn wounds (Gupta *et al*. 2005) and the lungs of the CF patients (Martin *et al*. 1993; Bakare *et al*. 2003). CF patients usually demonstrate imbalance in the microbial flora as a result of chronic use of antibiotics that leads to candidiasis (Burns *et al*. 1999). *C. albicans* and *P. aeruginosa* have an antagonistic interaction. *In vitro* analysis established that *C. albicans* biofilm formation and metabolism can be influenced by *P. aeruginosa* phenazines (Gibson *et al*. 2009; Morales *et al*. 2013). For instance, *P. aeruginosa* phenazines enhance ethanol production in *C. albicans* to stimulate biofilm formation in *Pseudomonas* (Chen *et al*. 2014). Another phenazine, methosulphate (PMS), can kill *Candida* within its biofilms (Morales *et al*. 2010; Morales *et al*. 2013). Several studies have also shown that *Pseudomonas* can inhibit *Candida* biofilm development *in vitro* (Holcombe *et al*. 2010; Bandara *et al*. 2010a; Bandara *et al*. 2010b; Reen *et al*. 2011; Bandara *et al*. 2013). Our previous studies further confirmed that this biofilm inhibition is *N*-acyl-L-Homoserine Lactone (HSL)-independent (Holcombe *et al*. 2010; Konstantinidou and Morrissey 2015). Collectively, these data suggest that *Candida* biofilm development and metabolism are intimately related with each other (Lindsay *et al.* 2014).

Interaction between *C. albicans* and *P. aeruginosa* is mainly based on signalling. Several studies suggest that *Pseudomonas* QS molecules are responsible for the signal-mediated communication (Hogan and Kolter 2002; McAlester *et al*. 2008; Deveau and Hogan 2011). *Pseudomonas* QS molecules that are known to modulate *Candida – Pseudomonas* interaction include two types of HSLs, *N*-(3-oxododecanoyl)-L-homoserine lactone (3O-C_{12}-HSL) (Hogan *et al*. 2004; McAlester *et al*. 2008) and *N*-butyryl-L-homoserine lactone (C_4-HSL) (Smith and Iglewski 2003), as well as HHQ (2-heptyl-4-quinolone) (Reen *et al*. 2011). *P. aeruginosa* can inhibit morphological switch of *C. albicans* from yeast to hyphae using these secreted chemicals. It was reported that *Pseudomonas* HSLs can inhibit the switch of *Candida* from yeast to filamentous growth (Hogan *et al*. 2004). However, QS molecule of *C. albicans*, farnesol, can limit the virulence of *P. aeruginosa* by blocking the production of *Pseudomonas* QS molecules and pyocyanin (Kerr *et al*. 1999; Cugini *et al*. 2007) and affecting motility (McAlester *et al*. 2008). *P. aeruginosa* pyocyanin and 1-hydroxyphenazine can prevent the growth of *C. albicans* (Kerr *et al*. 1999).

Other factors that affect the interaction between *C. albicans* and *P. aeruginosa* include iron availability (Purschke *et al*. 2012), bacterial cell wall lipopolysaccharides (LPS) (Bandara *et al*. 2010a; Bandara *et al*. 2013) and extracellular DNA (Sapaar *et al*. 2014).

1.7 Quorum Sensing

Natural polymicrobial communities include diverse species that interact with each other. Their interaction is regulated by the environmental sensing mechanism called autoinduction (Nealson 1977). Autoinducers are signalling molecules produced in response to cellular population density (Eberhard 1972). Given the population density and the dimensions of the environment, bacteria can alter or repress their gene expression. This stimulus and response system is known as quorum sensing (QS) (Eberhard *et al*. 1981). The QS system was firstly identified in the bacterium, *Vibrio fischeri*, a bioluminescent symbiont of the Hawaii squid (Nealson *et al*. 1970) and has been extensively studied in bacteria (Figure 4).

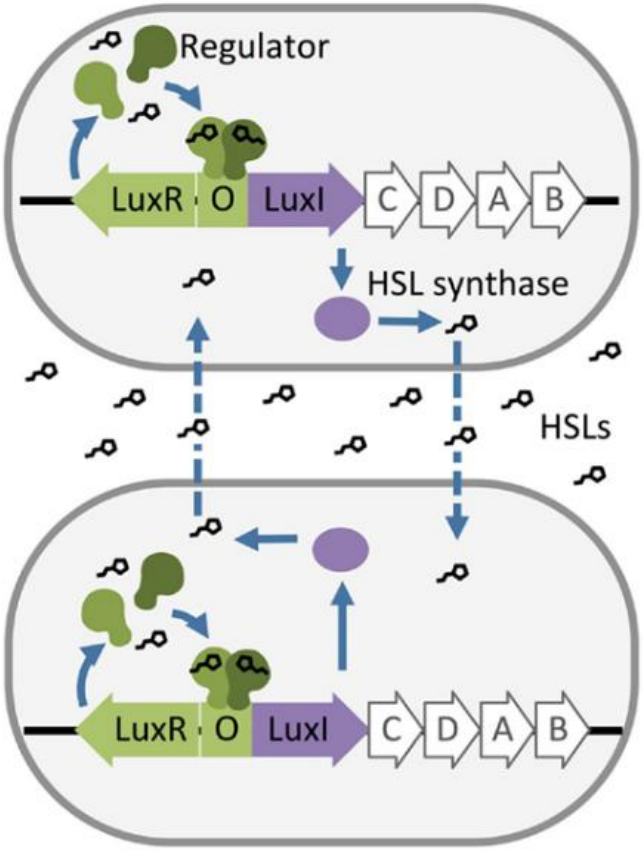

Figure 4| The structure of HSL (*N*-acyl-L-homoserine lactone) network in *Vibrio fischeri*. Luciferase expression system regulates expression of gene clusters such as LuxR, LuxI, C, D, A and B. O indicates an operator binding site for the regulator protein. This figure represents a part of a review article Figure (1) published by Davis *et al*. (2015).

Inter-kingdom communication across the prokaryotic and eukaryotic organisms is based on the QS molecules. For paradigm, a bacterial HSL (*N*-acyl-L-homoserine lactone) can regulate the gene expression in eukaryotes. The eukaryotes recognise and respond to the bacterial HSLs because their chemical structure considerably resembles eukaryotic hormones. This phenomenon is known as "global sensing" (Shiner *et al*. 2005; Hartmann and Schikora 2012).

1.7.1 *Pseudomonas aeruginosa* quorum sensing molecules

P. aeruginosa possesses a well-studied QS system (Pesci *et al*. 1997; Smith and Iglewski 2003; Bjarnsholt *et al*. 2010). The two known QS networks in *P. aeruginosa* are Las and Rhl (Pesci *et al*. 1997). Both systems employ a transcriptional activator and an autoinducer synthase to control gene expression (Pearson *et al*. 1997). *Pseudomonas* QS network regulates semantic functions such as virulence, protein secretion, swarming motility, 4-quinolone signalling as well as production of secondary metabolites, exoenzymes and exotoxins (Diggle *et al*. 2008).

Pseudomonas supernatants are rich in signalling molecules. PQS and its precursor HHQ (2-heptyl-4-quinolone) play an important role in *Pseudomonas* signalling. *P. aeruginosa* secrets the HSLs, *N*-(3-oxododecanoyl)-L-homoserine lactone (3O-C_{12}-HSL) and *N*-butyryl-L-homoserine lactone (C_4-HSL), that signal and regulate *C. albicans* behaviour. *P. aerugionsa* also produces phenazines such as phenazine methosulfate, phenazine-1-carboxylate and pyocyanin that can impair *C. albicans* biofilm development and metabolism (Gibson *et al*. 2009; Morales *et al*. 2013). Recently, coumarin (fragrance oil) was shown to inhibit *P. aeruginosa* phenazine production, biofilm development and swarming motility (Gutiérrez-Barranquero *et al*. 2015).

1.7.2 *Candida albicans* quorum sensing molecules

To promote biofilm formation and pathogenesis *C. albicans* also produces signalling molecules (Kruppa 2009). *C. albicans* QS molecules control the initiation of hyphae development via the protein degradation mediated with Ubr1 (Lu *et al*. 2014). *C. albicans* excretes among others two well-established QS molecules, farnesol and tyrosol (Figure 5). Farnesol is a water insoluble organic alcohol present in essential oils and used in perfumery. Farnesol represents a natural pesticide and insect pheromone. Additionally, farnesol is reported to possess antitumor (Joo and Jetten 2009) and antibacterial properties (Kromidas *et al*. 2006). For example, farnesol alters cell morphology and disrupts cell

membrane integrity of a bacterium *Acinetobacter baumannii* ultimately leading to the biofilm impairment (Kostoulias *et al*. 2015).

C. albicans produces farnesol in order to control its cell density (Figure 5A). Farnesol is secreted by solely the white cells of *C. albicans*. The principal biological function of farnesol is an inhibition of *C. albicans* filamentation (Hornby *et al*. 2001) by affecting cell amino acid incorporation (Braun 2005). Farnesol causes inflammation response by activation of the human innate immune cells (neutrophils and monocytes) simultaneously suppressing cellular adaptive immunity, differentiation of monocytes into immature dendritic cells (Leonhardt *et al*. 2015). Farnesol participates in complex signal transduction pathways of yeast to hyphae transition. Its mechanism of action involves activation of Ras/cAMP/PKA pathways (Davis-Hanna *et al*. 2008) and of a hyphal repressor, Tup1p (Kebaara *et al*. 2008), as well as of a Chk1p kinase (Kruppa *et al*. 2004). Farnesol has the ability to prevent biofilm formation in its late phase but cannot affect already existing hyphae (Hornby *et al*. 2001). Moreover, farnesol elevates the expression of genes regulating hyphal formation, drug resistance, cell wall maintenance and heat shock protein production (Cao *et al*. 2005). Westwater *et al*. (2005) also proposed a possible link between farnesol and the oxidative stress resistance. Collectively these data indicate that farnesol affects the dynamics of *C. albicans* morphogenesis (Martins *et al*. 2007).

Another QS molecule of *C. albicans*, tyrosol, represents a natural antioxidant derived from an aromatic phenethyl alcohol (Figure 5B). White wine and olive oil that are known to possess cardioprotective properties are rich in tyrosol (Samuel *et al*. 2008; Miró-Casas *et al*. 2003). Chen *et al*. (2004) showed that tyrosol is an autoregulatory QS molecule that delays fungal growth and stimulates germ tube formation in the diluted population of *C. albicans* (Chen *et al*. 2004). After more investigation it became evident that tyrosol also affects intermediate and early stages of the hyphae formation (Alem *et al*. 2006). In synopsis, *C. albicans* QS molecules, farnesol and tyrosol, have antagonistic function on filamentation.

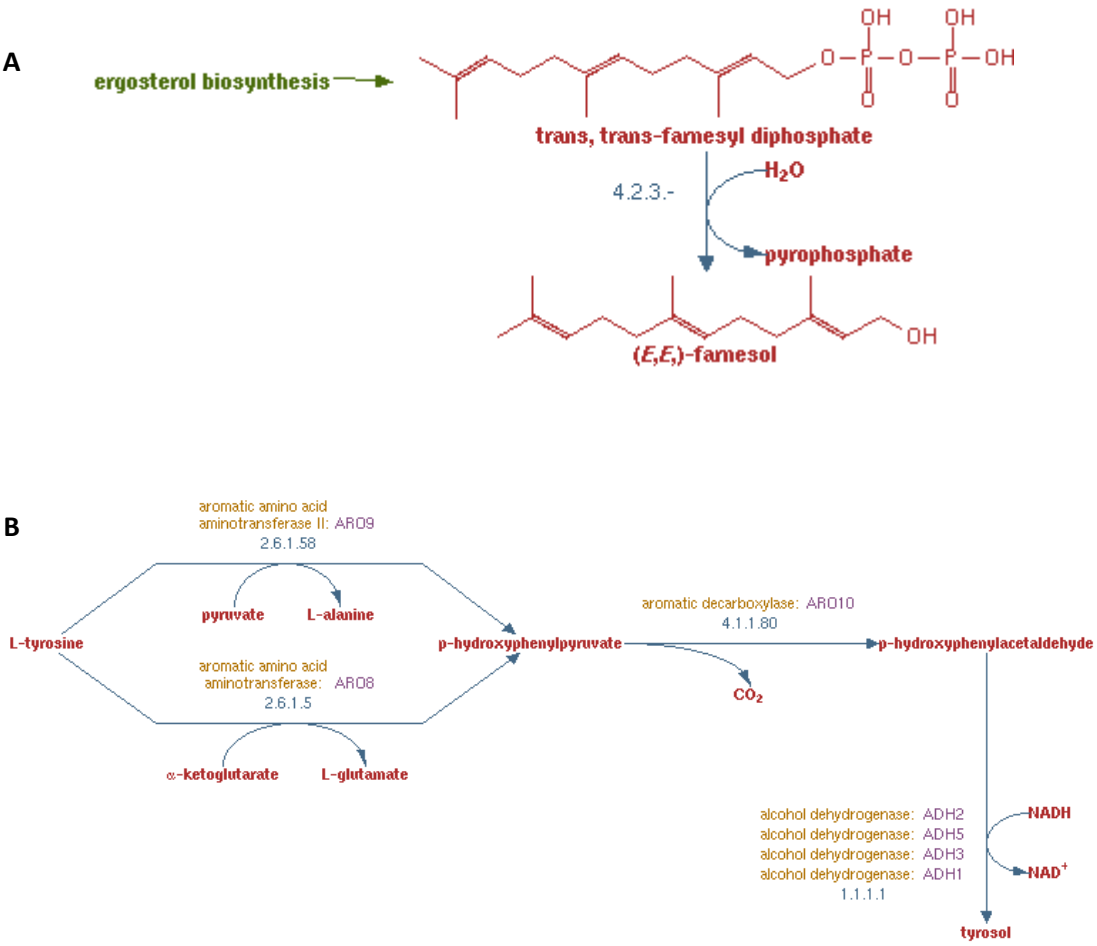

Figure 5| Quorum sensing (QS) molecule biosynthesis pathways in *C. albicans*. The Enzymes shown in bold indicate experimental evidence for their enzymatic activity. **A|** Biosynthesis pathway of farnesol. Farnesol is produced from farnesyl pyrophosphate, an intermediate in the ergosterol biosynthesis pathway and inhibits *C. albicans* filamentation and biofilm formation. **B|** Biosynthesis pathway of tyrosol. Both, constitutive (Aro8p) and inducible (Aro9p) aromatic aminotransferases can catalyze the initial reaction in tyrosine degradation. Tyrosol stimulates growth under dilute culture conditions and has a protective effect against human phagocytic cells. Tyrosol production is enhanced in biofilms compared to planktonic culture. Figures were obtained from *Candida* Genome database (CGD) (Arnaud *et al*. 2005).

Chapter 2

Co-occurrence of filamentation defects and impaired biofilms in *Candida albicans* protein kinase mutants

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Abstract

Pathogenicity of *C. albicans* is linked with its developmental stages, notably the capacity to switch from yeast-like to hyphal growth and to form biofilms on abiotic or biotic surfaces. To better understand the cellular processes involved in *C. albicans* development, a collection of 63 *C. albicans* protein kinase mutants was screened for biofilm formation in a microtitre plate assay. Thirty eight (38) mutants displayed some degree of biofilm impairment, with 20 categorised as 'Poor' biofilm formers. The morphology studies showed that all the 'Poor' biofilm formers were also defective in the switch from yeast to hyphae, establishing it as a primary defect of the impaired biofilms. Five genes, *VPS15, IME2, PKH3, PGA43 and CEX1*, were found to encode for proteins not previously reported to influence hyphal development or biofilm formation. To identify important processes for biofilm development we questioned the interactions of proteins/enzymes encoded by the biofilm-related genes using the STRING database. The STRING network analysis established that the PKA pathway, ribosome biogenesis and some aspects of the cell cycle are important for filamentation and biofilm development underlining the complexity of these processes.

2. Co-occurrence of filamentation defects and impaired biofilms in *Candida albicans* protein kinase mutants

2.1 Introduction

C. albicans has emerged as a serious nosocomial pathogen in part due to formation of drug resistant biofilms on indwelling medical devices such as urinary and vascular catheters. Hence, fungal biofilms have received a significant interest during the last decade (Chandra *et al*. 2001; López-Ribot 2005; Inglis *et al*. 2013). Biofilm development represents a complex phenomenon and *in vitro* it demonstrates three distinct phases. The early stage includes yeast cell attachment to the substrate as well as adhesion to the neighbouring cells. The mechanisms governing yeast attachment are important since they determine subsequent biofilm development. The intermediate phase involves pseudohyphal and hyphal filamentous growth. The maturation phase consists from two distinct processes, excretion of extracellular matrix (ECM) and blastospore dispersal (Chandra *et al*. 2001; Fox and Nobile 2012). This complex phenomenon is orchestrated by more than 1000 proteins including PKs, which are governed by 9 master regulators, Ndt80p, Efg1p, Brg1p, Bcr1p, Rob1p Tec1p, Flo8p, Gal4p and Rfx2p, that regulate signal transduction pathways at the genetic level (Nobile *et al*. 2012; Fox *et al*. 2015).

Biofilm development is regulated by diverse pathways but some well-characterised pathways are involved in the yeast to hyphae transition, Ras/cAMP/PKA (reviewed by Hogan and Sundstrom 2009); pseudohyphal induction, MAPK (reviewed by Srinivasa *et al*. 2012); and morphogenesis, calcium signalling pathways (Sanglard *et al*. 2003). For instance, inactivation of the transcription factors Cph1p (MAPK pathway) or Efg1p (Ras/cAMP/PKA pathway) can inhibit hyphal growth and biofilm development (Lo *et al*. 1997). Also, it was demonstrated that signalling via adenylyl cyclase is essential for hyphal development since cells defective in Cdc35p were unable to develop filaments (Rocha *et al*. 2001). The basic environmental factors characterized to induce fungal filamentation *in vitro* include body temperature (37^{o}C), neutral pH (7.0), and low cellular density (~10^{6} blastospores/ml), as well as presence of compounds such as serum (blood plasma without clotting factors), proline, glucose and *N*-acetyl-D-glucosamine (Mattia *et al*. 1982; Whiteway and Oberholzer 2004).

Protein phosphorylation is important for signal transduction processes with sequential activation of proteins often mediated by PKs. In this study, we took advantage of a set of PK

mutants to ask which PKs are required for biofilm formation in *C. albicans*. This collection has been the subject to many different screens showing that particular PK genes were responsible for cell wall regulation (Blankenship *et al*. 2010), cell-substrate attachment (Fanning *et al*. 2013), cell morphology (Blankenship *et al*. 2010), propolis-induced cell death (de Castro *et al*. 2013) and cell metabolism (Morales *et al*. 2013).

2.2 Materials and Methods

2.2.1 Yeast strains and growth conditions

The wild-type *C. albicans* strains used in this study were BWP17 (*ura3*Δ::λ*imm434/ura3*Δ::λ*imm434 his1*::*hisG/his1*::*hisG arg4*::*hisG/arg4*::*hisG*) (Wilson *et al*. 1999) and DAY286 (*ura3::λimm434 his1::hisG pARG4::URA3::arg4::hisG*) (Davis *et al.* 2002). The PK mutants of *C. albicans* are listed in Table 1. This kinase set was created by Aaron Mitchell's group (Blankenship *et al*. 2010) and obtained from the Fungal Genetics Stock Center (www.fgsc.net/candida/FGSCcandidaresources.htm). PK homozygous insertion mutants were created in BWP17 parental strain via Tn7-UAU1 cassette (Blankenship *et al*. 2010). The majority of the PK-coding genes were represented by double independent mutant strains but in some cases only a single mutant was available. In total, we examined 63 genes using 45 independent duplicate mutants and 18 single mutants. The initial FGSC collection was larger and included mutants deleted in ~80 PK/PK-related genes. Our reduced set comprised the mutants that, after delivery and culturing, we were able to verify by strain-specific PCR (see below).

Yeast strains were routinely cultured in standard rich medium containing 1% yeast extract, 2% peptone and 2% glucose (YPD). For biofilm and morphological analyses, the yeast strains were pre-grown in non-filament-inducing medium YNB (yeast nitrogen base), as described by McAlester *et al*. (2008) and Holcombe *et al*. (2010) with some adjustments. Briefly, filter-sterilized YNB salts without amino acids (Difco 291940) were supplemented with 0.2% glucose, 0.1% maltose and 0.16% filter-sterilized synthetic amino acid drop-out Leu^- (Kaiser Formedium DSCK052). For the induction of hyphal growth the strains were transferred to filament-inducing medium, YNBNP, which consisted from YNB supplemented with 2.5 mM *N*-acetyl-D-glucosamine (Sigma A8625) and 25 mM phosphate (sodium) buffer (pH 7).

2.2.2 PCR

DNA of wild-type *C. albicans* and PK mutants was extracted according to Hoffman (2001) protocol. The primers were designed with SnapGene (www.snapgene.com) and their sequences are listed in the Table 2. Strain-specific PCR was carried out using primers flanking the gene insertion sites listed in the Supplementary Table S1 of Blankenship *et al*. (2010) publication. Absence of the band indicated a mutant disrupted in the gene amplified by the corresponding primers (Appendix 2.5). All the PCR reactions were carried out

utilising a GoTaq Green Master Mix (Promega) according to the manufacturers' instructions.

2.2.3 Biofilm assay

A microtiter plate-based biofilm assay (XTT reductase salts) that measured metabolically active cells, was carried out as described by Ramage *et al*. (2001) and Holcombe *et al*. (2010). Briefly, the yeast strains were pre-grown in non-filament-inducing medium (YNB) overnight (30^{o}C) and diluted into filament-inducing medium (YNBNP) to A_{600}0.05. The diluted cultures (100 µL) were incubated for 1 h (37°C) in flat-bottomed 96-well polystyrene plates. After incubation, the attached cells were washed with fresh YNBNP medium twice, by inverting the plates carefully, to eliminate non-adhered cells. In order to induce biofilm formation the plates were incubated in the dark statically for 24 h (37°C). The next day, the biofilms developed on the bottom of the wells were washed with fresh YNBNP by careful pipetting. The XTT-menadione solution (100 µL), prepared as below, was added to the overnight cultures and incubated in the dark for 2 h (37^{o}C). Lastly, the dyed supernatants (80 µL) were transferred to a clean plate for the quantification at a wavelength of A_{490} nm.

For the preparation of the XTT solutions, 10 mM menadione (Sigma M5625) was dissolved in pure acetone and added (10 µL) to the XTT solution. The latter was prepared by dissolving 0.015 g of XTT powder (Sigma X4626) in 30 mL sterile dH_2O and filtered with 0.2 µm pore size filter.

To increase the statistical power of our experiments we carried out 2 independent screens and tested the mutants in triplicate with eight technical replicates (n = 24). Biofilms of the control, wild-type *C. albicans* DAY286, ranged from approximately A_{490}1 to A_{490}1.5. Thus, the mutants with biofilms above A_{490}1 and below A_{490}0.5 were assigned to the 'Good' and 'Poor' biofilm formers respectively. The remaining mutants were 'Moderate' biofilm formers. A distribution of each sample was assessed and the coincidence of the median and the mean indicated a Gaussian (normal) distribution of our data. All the statistical analysis of the biofilm assays were carried out in R Statistical Software (R Development Core Team 2013).

2.2.4 Morphological analyses

Morphological assays were carried out as described previously by Hogan *et al*. (2004) and McAlester *et al*. (2008). Briefly, the yeast cultures were pre-grown overnight in YNB broth

(30^{o}C) and diluted into YNBNP (A_{600}1.0) to assay their capacity to switch from yeast to filamentous growth. Before (0 h) and after 6 h and 10 h incubation (37^{o}C), the morphology of the mutants was examined microscopically. The images were captured digitally at a magnification x 400 via Zen2008 software with EX405 BP and Channel D by Zeiss Laser Scanning Microscope (LSM) 510.

2.2.5 Bioinformatics analyses

The protein sequences of *C. albicans* PKs were retrieved from the CGD (*Candida* Genome Database) (Arnaud *et al*. 2005), the analysis of the protein associations were carried out using the STRING v9.1 database (Search Tool for the Retrieval of Interacting Genes/Proteins) (Jensen *et al*. 2009), the GOs (Gene Ontologies) were obtained with the Blast2GO annotations (Conesa *et al*. 2005) and pathway analysis were carried out using KEGG pathway database (Kyoto Encyclopaedia of Genes and Genomes) (Kanehisa and Goto 2000).

Table 1| Protein kinase mutants used in this study.

Kinase Plate 1				Kinase Plate 2			
Position	**Orf19.**	**Gene**	**Updated**	**Position**	**Orf19.**	**Gene**	**Updated**
A7	orf19.4866	*CPP1*	*CPP1*	A1	orf19.7044	*RIM15*	*RIM15*
A8	orf19.4866	*CPP1*	*CPP1*	A2	orf19.7044	*RIM15*	*RIM15*
A10	orf19.5181	*NIK1*	*NIK1*	A3	orf19.5253		***YAK1***
B1	orf19.4084	*KIS1*	*KIS1*	A4	orf19.5253		***YAK1***
B2	orf19.4084	*KIS1*	*KIS1*	A5	orf19.2277	*TPK2*	*TPK2*
B3	orf19.469	*HST7*	*HST7*	A6	orf19.2277	*TPK2*	*TPK2*
B4	orf19.469	*HST7*	*HST7*	A8	orf19.7451	*FUN31*	*FUN31*
B9	orf19.5224	*Sc. PKH1*	***PKH2***	A9	orf19.3530	*CKA2*	*CKA2*
B10	orf19.5224	*Sc. PKH1*	***PKH2***	A10	orf19.3530	*CKA2*	*CKA2*
C1	orf19.451	*SOK1*	*SOK1*	A11	orf19.4297	*CKB2*	*CKB2*
C2	orf19.451	*SOK1*	*SOK1*	A12	orf19.4297	*CKB2*	*CKB2*
C3	orf19.1874	*Sc. MEK1*	***MEK1***	B1	orf19.4518		***YPL150W***
C4	orf19.1874	*Sc. MEK1*	***MEK1***	B2	orf19.4518		***YPL150W***
C7	orf19.2395	*Sc. IME2*	***IME2***	B3	orf19.2268	*RCK2*	*RCK2*
C8	orf19.2395	*Sc. IME2*	***IME2***	B4	orf19.2102	*CKB1*	*CKB1*
C9	orf19.3047	*Sc. SIP3*	***SIP3***	B7	orf19.844	*STE11*	*STE11*
C10	orf19.3047	*Sc. SIP3*	***SIP3***	B8	orf19.844	*STE11*	*STE11*
C11	orf19.794	*Sc. SSN3*	***SSN3***	B11	orf19.3049		***SPS1***
C12	orf19.794	*Sc. SSN3*	***SSN3***	B12	orf19.3049		***SPS1***
D1	orf19.4892	*TPK1*	*TPK1*	C1	orf19.2436	*SKY1*	*SKY1*
D2	orf19.4892	*TPK1*	*TPK1*	C2	orf19.846		***NNK1***
D3	orf19.5408	*Sc. HRK1*	***HRK1***	C3	orf19.846		***NNK1***
D4	orf19.5408	*Sc. HRK1*	***HRK1***	C4	orf19.7355	*SSN8*	*SSN8*
D5	orf19.223		***FPK1***	C5	orf19.3720		***BCY1***
D6	orf19.223		***FPK1***	C6	orf19.3720		***BCY1***
D9	orf19.1196	*Sc. PKH1*	***PKH3***	C7	orf19.2910	*PGA43*	*PGA43*
D10	orf19.1196	*Sc. PKH1*	***PKH3***	C9	orf19.4002		***DUN1***
D11	orf19.835	*IES1*	*IES1*	C10	orf19.428	*IKS1*	*IKS1*
D12	orf19.835	*IES1*	*IES1*	C11	orf19.428	*IKS1*	*IKS1*
E1	orf19.469	*HST7*	*HST7*	C12	orf19.1341	*PRR2*	*PRR2*
E2	orf19.469	*HST7*	*HST7*	D4	orf19.6243	*VPS34*	*VPS34*
E3	orf19.4084	*KIS1*	*KIS1*	D7	orf19.6889	*MKK2*	*MKK2*
E4	orf19.4084	*KIS1*	*KIS1*	D8	orf19.6889	*MKK2*	*MKK2*
E5	orf19.2341	*HNT1*	*HNT1*	D11	orf19.1341	*PRR2*	*PRR2*
E6	orf19.2341	*HNT1*	*HNT1*	D12	orf19.1341	*PRR2*	*PRR2*
E7	orf19.35	*Sc. SKY1*	***SKY2***	E1	orf19.130	*VPS15*	*VPS15*
E8	orf19.35	*Sc. SKY1*	***SKY2***	E2	orf19.130	*VPS15*	*VPS15*
E9	orf19.4001	*Sc. MSS2*	***MSS2***	E3	orf19.2222	*YCK3*	*YCK3*
E10	orf19.4001	*Sc. MSS2*	***MSS2***	E7	orf19.1283	*MEC1*	*MEC1*
E11	orf19.4867	*SWE1*	*SWE1*	E9	orf19.7510	*KIN2*	*KIN2*
E12	orf19.4867	*SWE1*	*SWE1*	E10	orf19.7510	*KIN2*	*KIN2*
F1	orf19.7281	*Sc. PKP1*	***PDK2***	E11	orf19.5911	*CMK1*	*CMK1*
F2	orf19.7281	*Sc. PKP1*	***PDK2***	E12	orf19.5911	*CMK1*	*CMK1*
F3	orf19.3744		***CEX1***	F1	orf19.7164		***ENV7***
F5	orf19.4144		***MCP2***	F2	orf19.7164		***ENV7***
F6	orf19.4269			F5	orf19.895	*HOG1*	*HOG1*
F9	orf19.4890	*CLA4*	*CLA4*	F6	orf19.895	*HOG1*	*HOG1*
F10	orf19.4890	*CLA4*	*CLA4*	F7	orf19.5162	*BCK1*	*BCK1*
F11	orf19.4308	*HSL1*	*HSL1*	F8	orf19.5162	*BCK1*	*BCK1*
F12	orf19.4308	*HSL1*	*HSL1*	F9	orf19.7652	*CKA1*	*CKA1*
G1	orf19.4432	*KSP1*	*KSP1*	F10	orf19.7652	*CKA1*	*CKA1*
G2	orf19.4432	*KSP1*	*KSP1*	F11	orf19.460	*CEK2*	*CEK2*
G5	orf19.7510	*KIN2*	*KIN2*	F12	orf19.460	*CEK2*	*CEK2*
G6	orf19.7510	*KIN2*	*KIN2*				
G7	orf19.2678		***BUB1***				
G8	orf19.6913	*GCN2*	*GCN2*				
G9	orf19.6913	*GCN2*	*GCN2*				
G10	orf19.5068	*IRE1*	*IRE1*				
G11	orf19.5068	*IRE1*	*IRE1*				
H1	orf19.3854		***SAT4***				
H2	orf19.5357	*AKL1*	*AKL1*				
H5	orf19.7510	*KIN2*	*KIN2*				
H6	orf19.7510	*KIN2*	*KIN2*				
H7	orf19.5224	*PKH1*	***PKH2***				
H8	orf19.5224	*PKH1*	***PKH2***				
H9	orf19.3751		***KIN4***				
H10	orf19.3049		***SPS1***				
H11		DAY286					

Mutants with updated names are given in **bold** type.

Table 2| Primers used in this study.

Forward	Sequence (3' – 5')	Reverse	Sequence (3' – 5')
BUD32_F	ATGACAGATCACCTAATTGCTAAAGTAC	BUD32_R	TCAACCCAACATACTTCTTTTTCTTCC
CKA2_F	AGTTTTCCAAGGTGTCAATGTTTT	CKA2_R	TTGAAAAATGGATGTTCCATTGCC
CLA4_F	CCTTCATCTCAACAACAGCAACAA	CLA4_R	TCCTTTTGTTTAACCACTTCAGGT
CTK3_F	TTCACACAGGAAACAGCTATGACCATGAT TACGCCAAGCTcatcacattggtcgtcctggaaatc	CTK3_R	TCGACCATATGGGAGAGCTCCCAACGCGT TGGATGCATAGctattgatgaagcaaactacgagtatgtgaac
GIN4_F	CGTTTGGATAAAGCTGGATTGGC	GIN4_R	GGAACTTTGGATTTTGGTCTTTGCC
IME2_F	AAGTGCAACTATTTCCATCGTGAC	IME2_R	CTTGTAGCTTTCATTCCCAGAACT
KIN3_F	ATGTCGATTATCGATGAATATGAATC	KIN3_R	TTATCGGTACTTACTTATATACTCAAACT
KIS1_F	TGCTCAGTCCAAAATCTACAAATC	KIS1_R	CATTGCTTTCATCATCATGGTATC
KIS1_FF	TGAATCAGCAACAGCATTCACAAT	KIS1_RR	ATTCAACACAACGTGGTTTGGAAT
MSS2_F	TCAAATGCAACGAAAGCGACTATT	MSS2_R	TCCTGAACTTGATGAAATTTCCCA
NIK1_F	GGTTACCTCGGAGTATGGATCCG	NIK1_R	GAATAGAATGATGGACCAAAACCAACGG
orf19.3744_F	CCTCCTAAGATGTCAGCGTCCG	orf19.3744_R	GTTGATAGTGTTTCTTGACGTCCTGGG
PGA43_F	GCCCTAGCACGAATTATTGATCCAG	PGA43_R	GGCTTGACATTGTGGATACTTCCG
PKH3_123_F	GAACATCTACAGAACTTATCTATCCAGCC	PKH3_123_R	GGAATATGATCCTTCTCCTATTTTCGC
PKH3_749_F	CCGGAATTACTTAAGCACAATATATGCG	PKH3_749_R	CCACCTTGATGACATGATATGTGGG
PRK1_1145_F	CACCTCTAAACCAAAGACAGATCCG	PRK1_1145_R	CCCTGAGAATATATTCTTGGTGTATTGCC
PRK1_485_F	GTATCAGGTGACTATAGGTGTGGCC	PRK1_485_R	GGTGGTAAATAATTTACCGACGAGCC
PTK2_F	CAATGGATATGTTGTTTGACGACCC	PTK2_R	GAATGTACCTCTTCTAGATGGCGC
RIO2_F	ATGCACCCAAAAAAAAAAAAAAAGAAG	RIO2_R	CTATTCATCGAGTATATAATTTCCTAGCT
SAT4_F	CCTTCCCCTTCTAATGGAACTACCG	SAT4_R	CAGTAGGGGTATTGACAGAAGTCGG
SSN3_F	AATGTTGGGATATCTCAACCATCA	SSN3_R	GGAATTGGTTTAAAATCAGGATGC
SSN8_F	CCTCCTCATACTATAGCGGTGGC	SSN8_R	CTTGACCAAGAACTTGAGTTTCTTGGG
VPS15_F	TAAACATCAATACCTGCAACAGCA	VPS15_R	TACCACCGTCATTCTTTGTCTCAA
VPS34_F	GCTTTTTGAGGAAATTAGCAGTTG	VPS34_R	CGGAAATTGGACTAGTAGCCAATA

2.3 Results

2.3.1 Identification of the protein kinases involved in *Candida albicans* biofilm formation

To define the genetic control of biofilm development in *C. albicans*, a collection of 63 homozygous insertion mutants disrupted in PK and PK-related genes (Blankenship *et al*. 2010) was screened twice independently for altered biofilm formation. A pipeline was developed to carry out the screen and to classify the mutants into 5 classes based on their biofilm formation (Figure 1). A 96-well polystyrene microtitre plate-based biofilm assay (XTT reductase) was used to represent the abiotic surface of indwelling medical devices that serve as a convenient substrate for *Candida* biofilm development *in vivo*. The variability in the XTT assay illustrated the importance of the two independent screens and of a large sample size (n = 24), which enhanced the statistical power of the results. The biofilm assay revealed that 38 mutants (>60%) had reduced biofilm development and were classified as 'Poor' (sub-classes 'Very poor' and 'Poor') or 'Moderate' biofilm formers. Data for the biofilm formation of the mutants classified as 'Poor' and 'Moderate' are shown in Figures 2 (A, B) and 3 (A) respectively. The biofilm formation levels of the remaining 25 mutants were not significantly different from that of the control (DAY286), hence they were classified as 'Good' (sub-classes 'Good' and 'Very good') biofilm formers (Figure 3 (B and C); Table 3). Our results were consistent with the published literature, since more than 60% (23/38) of biofilm-defective mutants had deletions in the genes previously known to be required for biofilm formation and/or filamentation (see footnotes at the end of Table 3). Fifteen mutants (5 poor and 10 moderate) represented genes in *C. albicans* that were not previously directly implicated in filamentation or biofilm formation (Table 3). The CGD descriptions of all the *C. albicans* PK mutants used in this study can be found in the Appendix 3.5 (Chapter 3).

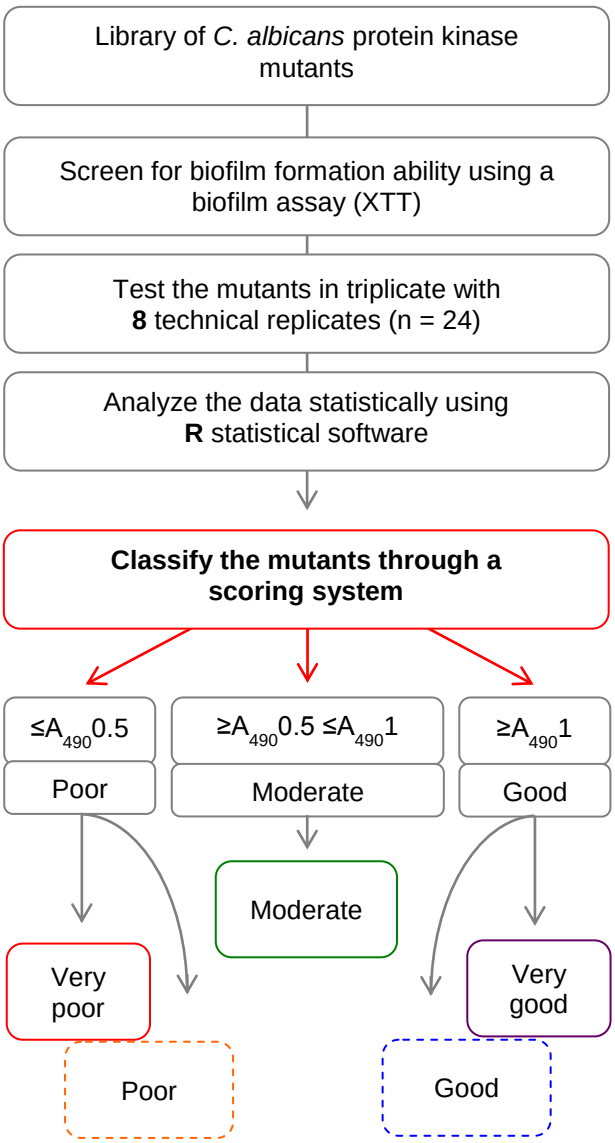


Figure 1| Workflow for the categorisation of *C. albicans* protein kinase mutants based on their biofilm formation ability. Sixty-three (63) PK mutants, obtained from the Fungal Genetics Stock Centre, were screened to identify strains with impaired biofilms. Two (2) independent screens were carried out using a microtiter plate-based biofilm assay (XTT), which measured the metabolic activity of the cells. Biofilms of the control, wild-type *C. albicans* DAY286, ranged from approximately $A_{490}1$ to $A_{490}1.5$. Hence, the mutants that developed biofilms above $A_{490}1$ and below $A_{490}0.5$ were assigned to the 'Good' and the 'Poor' biofilm formers respectively. The rest exhibited moderate biofilm formation trends that fluctuated between $A_{490}0.5$ and $A_{490}1$. For more detailed analysis, the mutants that showed different biofilm formation ability were classified into the intermediate classes, 'Very Poor / Poor' and 'Very Good / Good'.

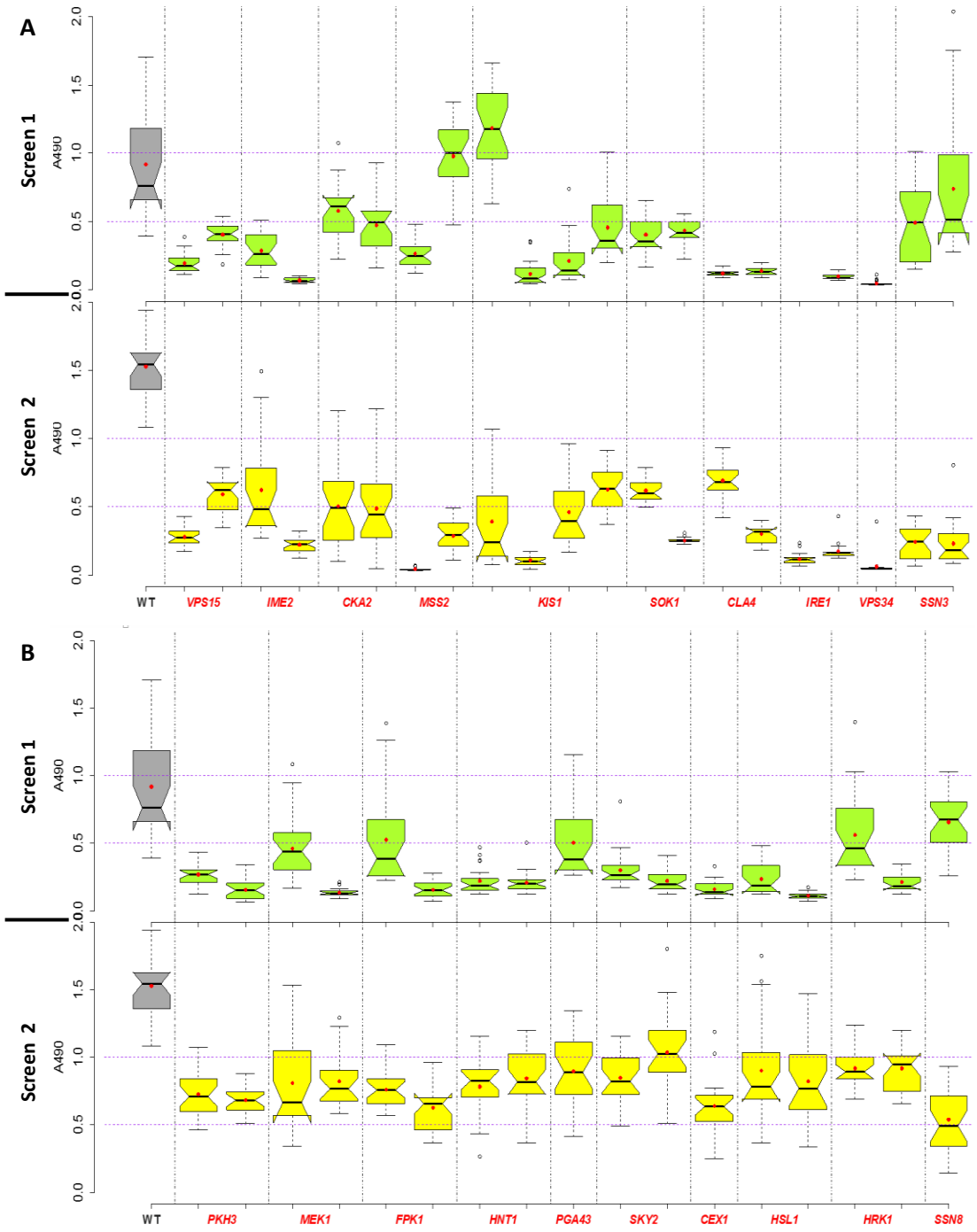
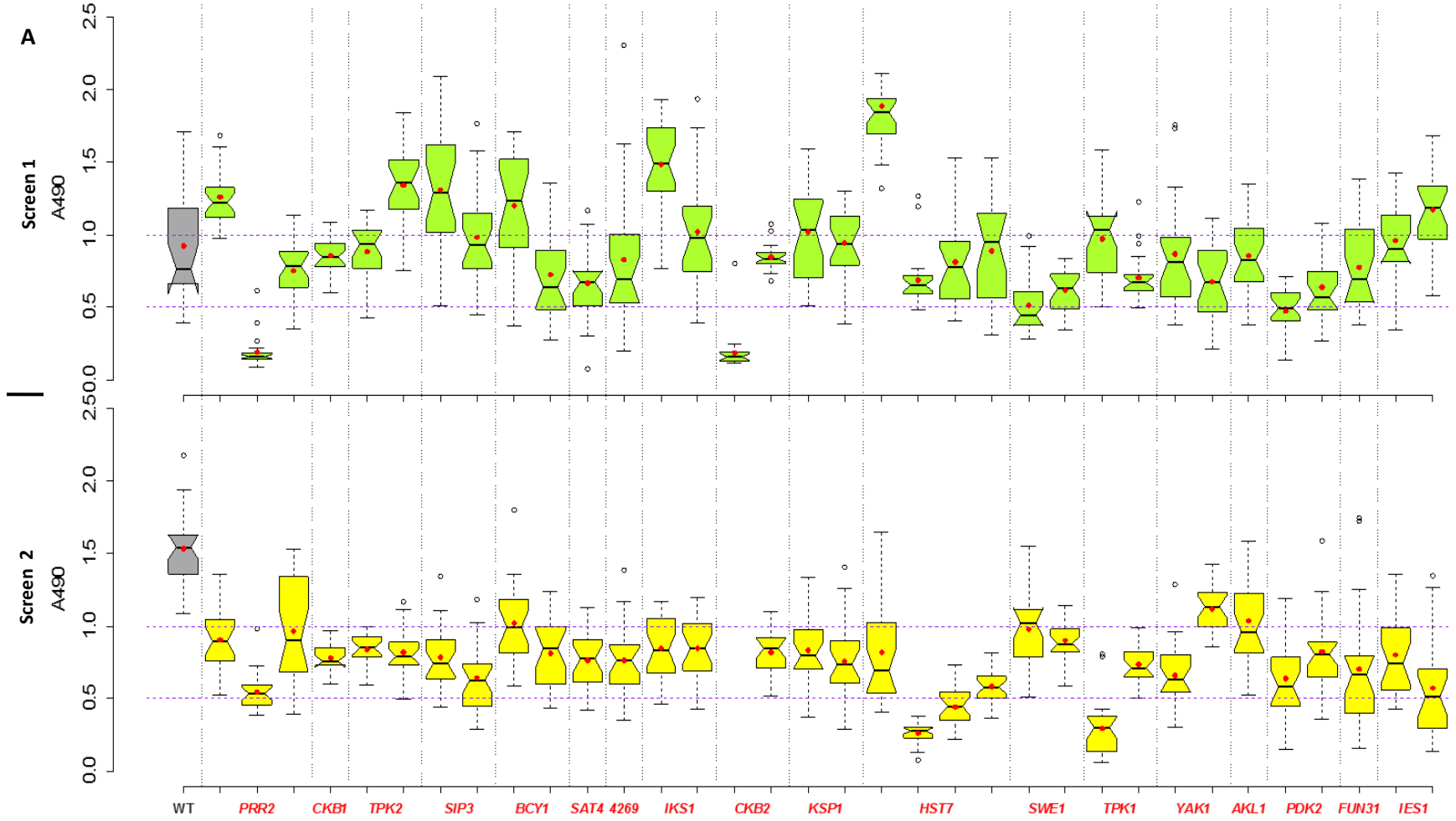


Figure 2| Biofilm formation of the 'Poor' biofilm former mutants. The metabolic activity of the biofilms was evaluated via the XTT reduction assay. **A|** and **B|** show 'Very poor' and 'Poor' biofilm formers respectively. Two independent screens were carried out and labelled as screen 1 (green bars) and screen 2 (yellow bars). Each screen included a wild-type strain DAY286 (gray box). This control is important because of variation in the efficiency of XTT between assays. Most mutants were present as two independent mutants in the collection, though one (*KIS1*) was present four times and several (*VPS34, PGA43, CEX1* and *SSN8*) only

once. Sample size equals to 24 observations (n = 24). 50% of the observations fall into the box. The bottom and the top of the box correspond to the 1st and the 3rd quartiles respectively. The notch shows the median confidence interval and the middle line inside the box indicates the median. The upper and the lower limits demonstrate the maximum and the minimum observations. The red dot shows the mean and the white circle an outlier. Horizontal dashed line illustrates a threshold defined in Figure 1.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Screen 2
A490
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1.0
1.5
2.0
2.5
WT
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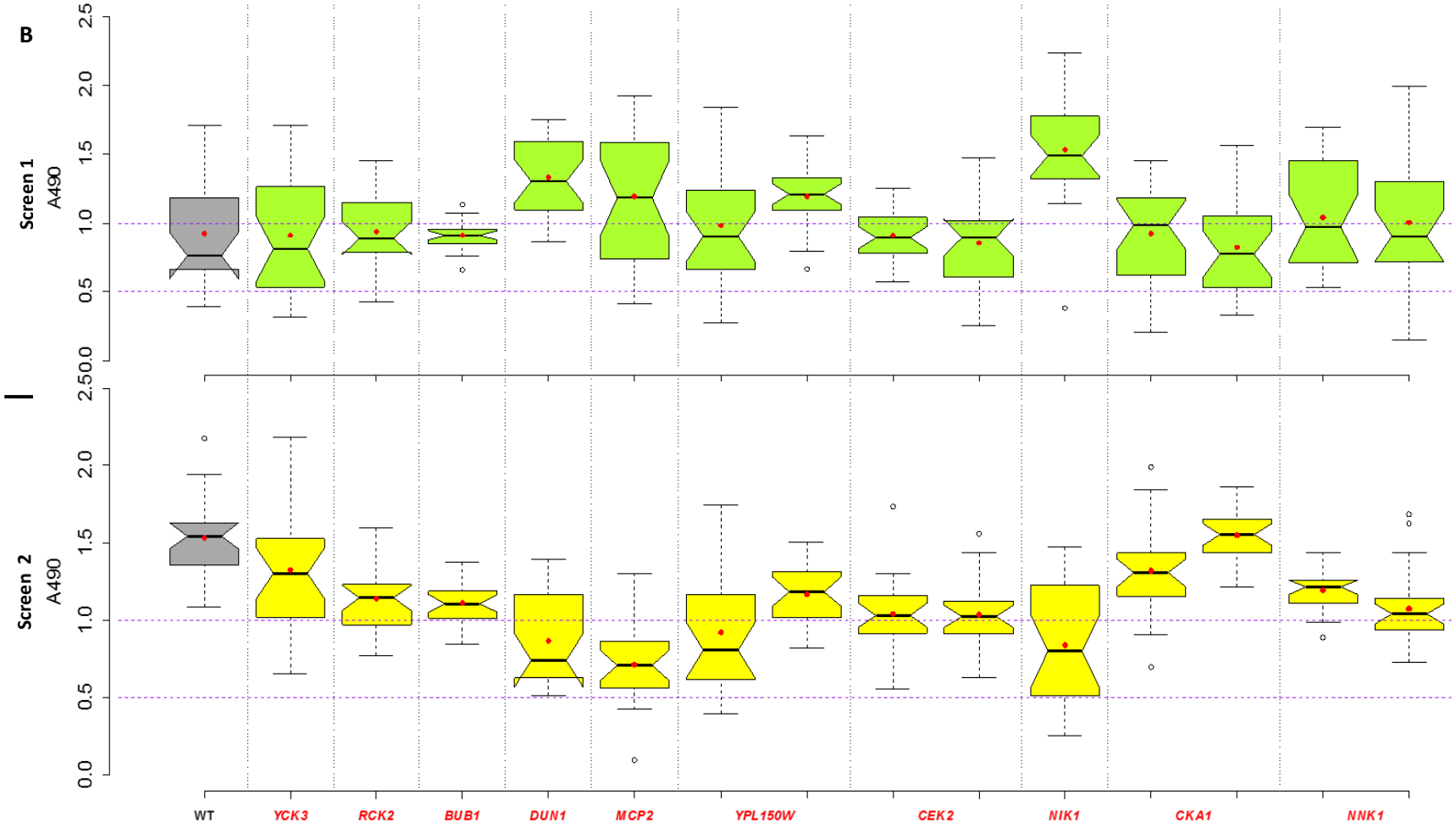
CKB1
TPK2
SIP3
BCY1
SAT4 4269
IKS1
CKB2
KSP1
HST7
SWE1
TPK1
YAK1
AKL1
PDK2
FUN31
IES1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Screen 2
A490
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0.5
0.0
WT
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RCK2
BUB1
DUN1
MCP2
YPL150W
CEK2
NIK1
CKA1
NNK1


C
Screen 1
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Screen 2
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0.5
1.0
1.5
2.0
2.5
WT
MEC1
SKY1
SPS1
KIN4
CPP1
BCK1
PKH2
CMK1
MKK2
GCN2
RIM15
ENV7
KIN2
STE11
HOG1

Figure 3| **Biofilm formation of the 'Moderate' and 'Good' biofilm formers.** The metabolic activity of the biofilms was evaluated via the XTT reduction assay. **A**|, **B**| and **C**| show the 'Moderate', 'Good' and 'Very good' biofilm formers respectively. Two independent screens were carried out and the results are presented as follows: top panel, green boxes – screen 1; bottom panel, yellow boxplots – screen 2. Each screen included a wild-type *C. albicans* DAY286 (gray box). This control is important because of variation in the efficiency of XTT between assays. Most mutants were presented as two independent mutants in the collection, though some were presented several times (e.g. *PKH2, KIN2*) and others (e.g. *MEC1, SKY1*) only once. Sample size equals to 24 observations (n = 24). 50% of the observations fall into the box. The bottom and the top of the box correspond to the 1^{st} and the 3^{rd} quartiles respectively. The notch shows the median confidence interval and the middle line inside the box indicates the median. The upper and the lower limits demonstrate the maximum and the minimum observations. The red dot shows the mean and the white circle an outlier. Horizontal dashed line illustrates a threshold defined in Figure 1.

Table 3| **Classes of *C. albicans* protein kinase mutants based on their biofilm development.** PK mutants (63) were assessed through a biofilm assay (XTT) and classified into different categories according to the biofilms they developed. Many mutants (38) showed impaired biofilm development and more than a half of them (20) were poor biofilm formers. Genes firstly associated with biofilms under our assay conditions are given in **bold** type.

Poor		Moderate	Good	
Very poor	**Poor**		**Good**	**Very good**
***VPS15* (C6_01190C)**[f]	***PKH3* (C6_00350W)**[f]	***PRR2* (C7_03340C)**[f]	*YCK3* (C2_08270C)[a, f, g, h]	*MEC1* (C5_04060C)[a, d]
***IME2* (CR_03290C)**	*MEK1* (C2_07530C)[d]	***CKB1* (C2_00300C)**[a, g]	*RCK2* (C2_07130C)[a, d, f]	*SKY1* (C1_06090C)[a, b]
CKA2 (C2_04980C)[d, f, g]	*FPK1* (C2_08860W)[b, f, h]	*TPK2* (C2_07210C)[b, c, d]	*BUB1* (C4_03120C)[a]	*SPS1* (C1_03470C)[f]
MSS2 (C5_05090W)[d, g]	*HNT1* (C1_10780C)[b]	***SIP3* (C1_03450C)**[g]	*DUN1* (C5_05100C)[a]	*KIN4* (CR_02210W)[a, f]
KIS1 (C2_09230C)[d, g]	***PGA43* (C4_06260W)**[a]	***BCY1* (CR_02460W)**[h]	*MCP2* (C5_01490C)[a, h]	*CPP1* (C1_10000C)[d, g]
SOK1 (CR_06000W)[b, d, f]	*SKY2* (C2_06600W)[b]	*SAT4* (CR_06040W)[a, b, f, g]	*YPL150W* (C2_04360W)[h]	*BCK1* (C7_02990W)[g]
CLA4 (C1_10210C)[d, g]	***CEX1* (CR_02250C)**[a, h]	**orf19.4269 (C5_02560C)**[a]	*CEK2* (CR_05940W)[d]	*PKH2* (C1_12410C)[f, g]
IRE1 (C1_07970C)[b, d, f, g]	*HSL1* (C5_02840C)[b, d, g]	*IKS1* (C1_05370C)[b]	*NIK1* (C7_02800W)[a, d]	*CMK1* (C3_04550C)
VPS34 (C1_06680W)[a, d, g]	*HRK1* (C3_00550C)[c]	***CKB2* (C5_02760W)**[f, g]	*CKA1* (CR_10660W)	*MKK2* (C2_05780C)[g]
SSN3 (C2_04260W)[e]	*SSN8* (C3_05740C)[a, e]	***KSP1* (C1_07380C)**	*NNK1* (C2_03760C)[h]	*GCN2* (C7_01330C)[d]
		HST7 (CR_03900W)[d, f, g]		*RIM15* (C7_00740W)[d, g]
		SWE1 (C1_10010C)[b, d, g]		*ENV7* (C7_04110W)[h]
		TPK1 (C1_10220C)[b, d, f, g]		*KIN2* (CR_00260W)[g]
		YAK1 (C1_12120W)[d, g]		*STE11* (C2_03770C)[d, f]
		***AKL1* (C2_10750C)**[a, f]		*HOG1* (C2_03330C)[d]
		***PDK2* (CR_08860W)**[f]		
		FUN31 (C3_06620W)[a, b, f]		
		***IES1* (C2_03900C)**		

[a] Single mutant strain available for testing.
[b] Linked with biofilms (Nobile *et al*. 2012; Inglis *et al*. 2013; Bandara *et al*. 2013).
[c] Down in biofilms (Nobile *et al*. 2012).
[d] Linked with filamentous growth (Blankenship *et al*. 2010; Inglis *et al*. 2013; Goyard *et al*. 2008).
[e] Wrinkled colony formation (Lindsay *et al*. 2014).
[f] Altered cell-surface adherence (Fanning *et al*. 2012).
[g] Cell wall stress phenotype (Blankenship *et al*. 2010).
[h] Gene name indicates an ortholog in *Saccharomyces cerevisiae* (CGD 2010).

2.3.2 Identification of the protein kinases involved in *Candida albicans* filamentous growth

Filamentation is an important aspect of biofilm formation, therefore the 20 poor biofilm formers (listed as 'Very poor' and 'Poor' in Table 3), were assayed for their ability to switch from yeast to hyphal growth (Figures 4, 5 and 6). Generally, it was observed that some mutants failed to filament while others did but not as well as the wild-type, establishing the filamentation as a primary defect. The morphology studies showed that all the poor biofilm forming mutants were also defective in the switch from yeast to hyphae, revealing a complete overlap between these processes. This analysis showed that 5 genes, *VPS15, IME2, PKH3, PGA43* and *CEX1*, which were not previously known to have these functions, were required for both filamentation and biofilm formation under our assay conditions (Figure 4). More specifically, the mutants disrupted in *VPS15* and *IME2* genes failed to filament, *PKH3* and *PGA43* deleted mutants displayed delayed filamentation and *CEX1* knock-out was less able to form hyphae compared to the wild-type *C. albicans* (Figure 4).

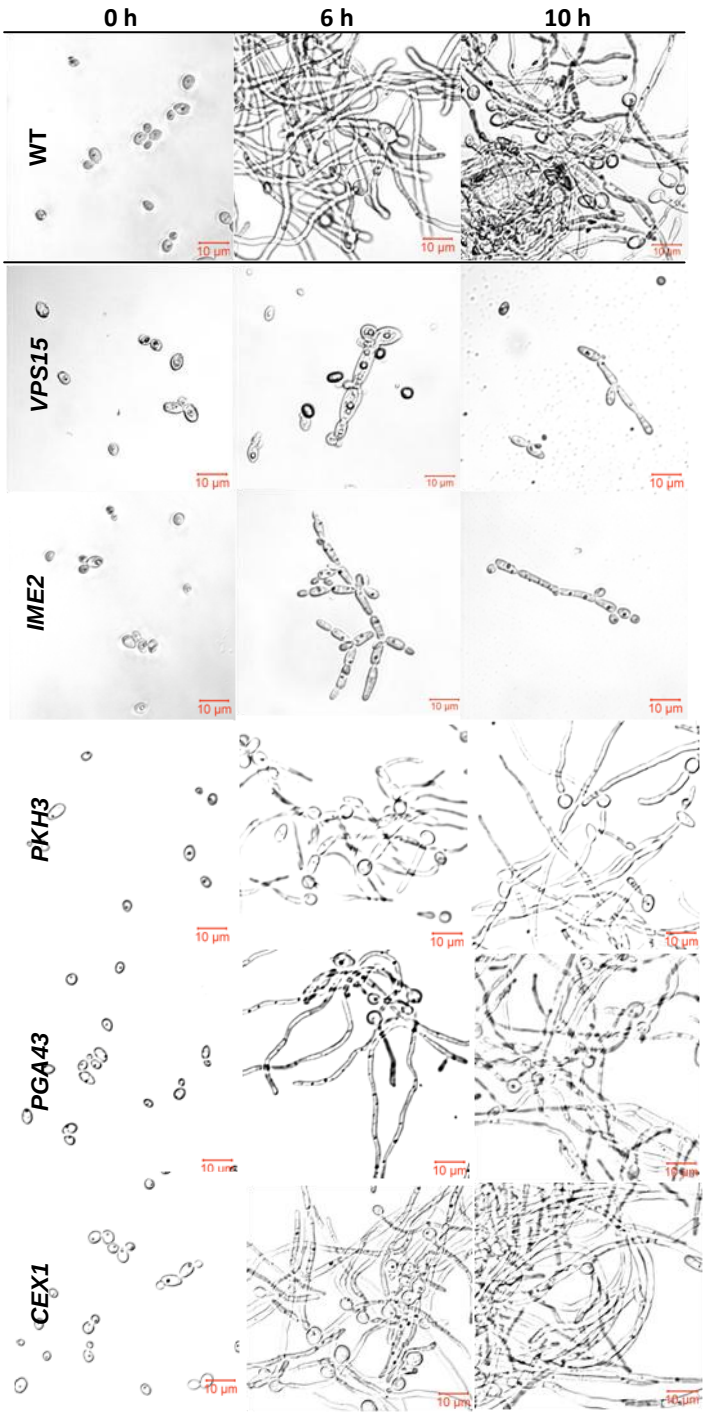
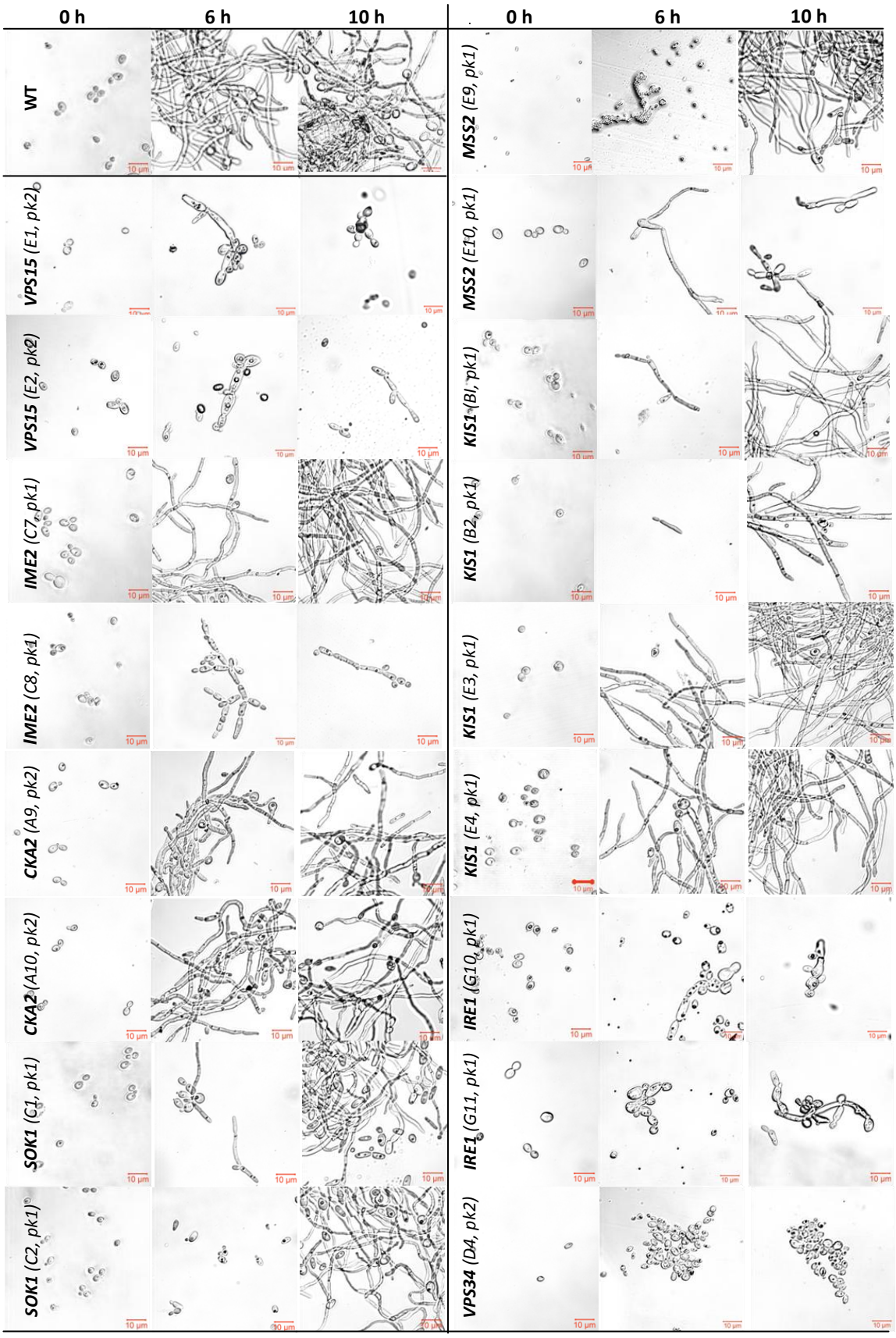


Figure 4| Morphology of the 'Poor' biofilm formers reveals their defective filamentation. The PK mutants of *C. albicans* were pre-grown in YNB overnight (30°C) and transferred to filament-inducing medium YNBNP (37°C). The morphology of the mutants and of a control *C. albicans* DAY286, was examined at 0, 6 and 10 h time points. The representative images were captured digitally via a Zeiss Laser Scanning Microscope (LSM) 510 using Zen 2008 software with EX405 BP, DICII and Channel D at a magnification x 400. The scale bar corresponds to 10 µm. Data are extracted from Figures 5 and 6.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6 h
10 h
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VPS15 (E2, pk2)
IME2 (C7, pk1)
IME2 (C8, pk1)
CKA2 (A9, pk2)
CKA2 (A10, pk2)
SOK1 (C1, pk1)
SOK1 (C2, pk1)
MSS2 (E9, pk1)
MSS2 (E10, pk1)
KIS1 (B1, pk1)
KIS1 (B2, pk1)
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VPS34 (D4, pk2)

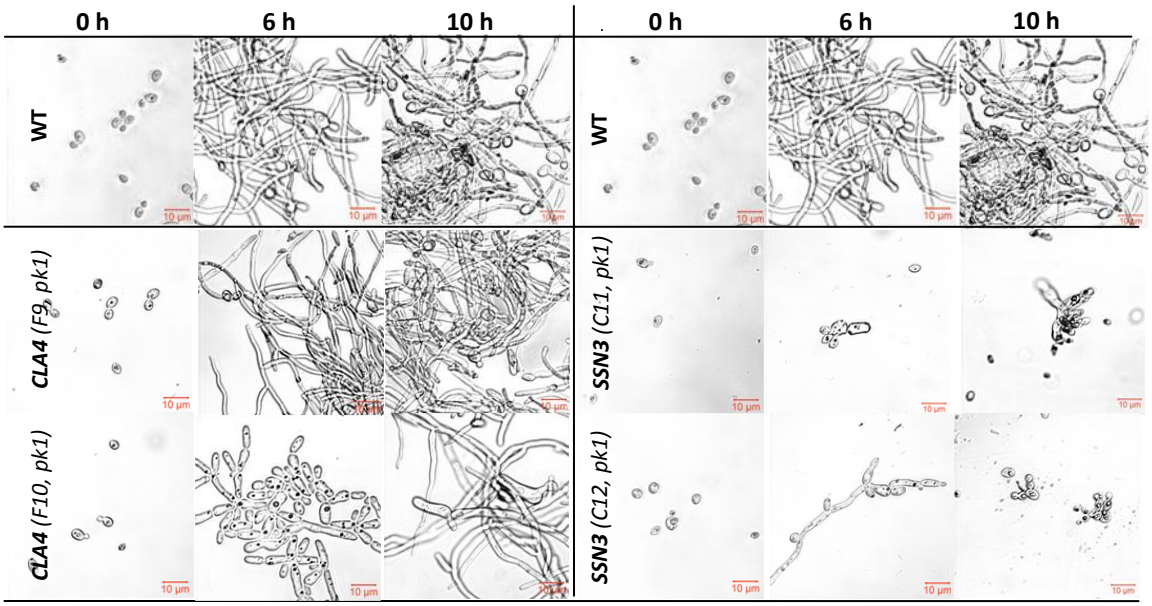


Figure 5| Morphology of the 'Very poor' biofilm formers reveals their defect in the switch from yeast to filamentous growth. The PK mutants of *C. albicans* were pre-grown in YNB overnight (30°C) and transferred to filament-inducing medium YNBNP (37°C). The morphology of the mutants and of a control *C. albicans* DAY286, was examined at 0, 6 and 10 h time points. The position of the mutant on the PK plate is indicated in parentheses. The representative images were captured digitally via a Zeiss Laser Scanning Microscope (LSM) 510 using Zen 2008 software with EX405 BP, DICII and Channel D at a magnification x 400. The scale bar corresponds to 10 µm.

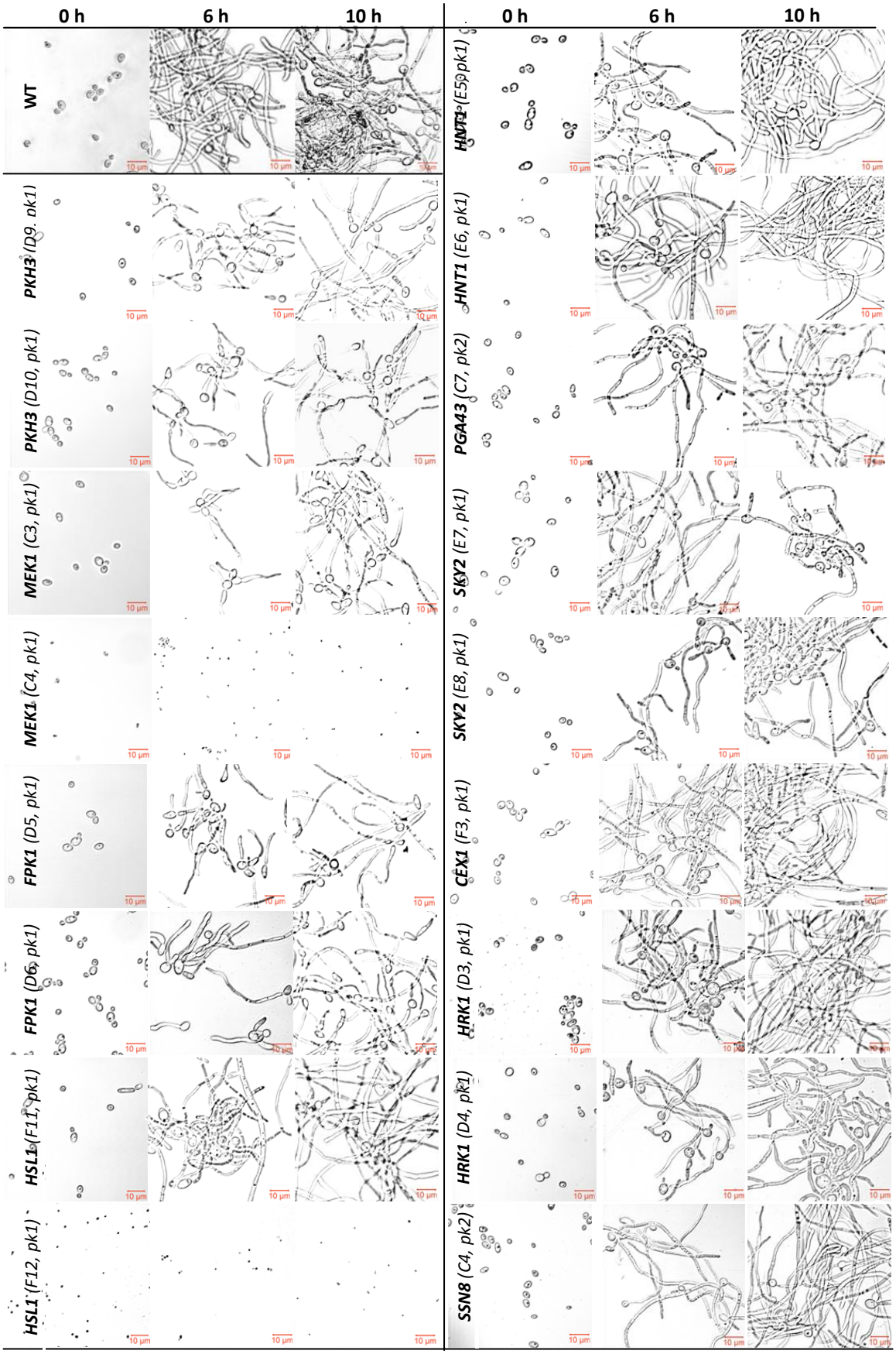

0 h
6 h
10 h
WT
PKH3 (D9, pk1)
PKH3 (D10, pk1)
MEK1 (C3, pk1)
MEK1 (C4, pk1)
FPK1 (D5, pk1)
FPK1 (D6, pk1)
HSL1 (F11, pk1)
HSL1 (F12, pk1)
HNT1 (E5, pk1)
HNT1 (E6, pk1)
PGA43 (C7, pk2)
SKY2 (E7, pk1)
SKY2 (E8, pk1)
CEX1 (F3, pk1)
HRK1 (D3, pk1)
HRK1 (D4, pk1)
SSN8 (C4, pk2)
10 µm

Figure 6| The 'Poor' biofilm former mutants are defective in the switch from yeast to filamentous growth. The PK mutants of *C. albicans* were pre-grown in YNB overnight (30^{o}C) and transferred into filament-inducing media YNBNP (37^{o}C). *C. albicans* DAY286 was used as a control. The position of the mutant on the PK plate is indicated in parentheses. The morphology of the mutants was examined at three time-points, 0 h, 6 h and 10 h, utilizing Zeiss Laser Scanning Microscope (LSM) 510. The representative images were captured digitally using Zen 2008 software with EX405 BP, DICII and Channel D at a magnification x 400. Scale bar corresponds to 10 µm.

2.3.3 STRING network analyses

To identify important processes for biofilm development we questioned the interactions of proteins/enzymes encoded by the 38 biofilm-related genes using a STRING database (Figure 7). STRING is a powerful database that quantitatively integrates experimental data, computational prediction methods and published literature to produce an interaction network (Jensen *et al*. 2009). Protein sequences were retrieved from CGD (www.candidagenome.org) and entered into the STRING network generation program (www.string-db.org). The resulted networks, which were analysed using a K-means option, showed that biofilm-related proteins were assigned into three main clusters (Figure 7). The 38 proteins were also analysed for their function in the KEGG annotated pathways (Table 4). The proteins of the clusters mostly shared similarity in function or occurrence in the same pathway. A list of the putative functional partners can be found in the Table 5. Combined, these analyses revealed three main clusters: the first (on the left) corresponded to the genes of the Protein Kinase A (PKA) pathway; the second (centre) to the elements of cell cycle; and the third (right) to vacuolar function (Figure 7). Some genes (11) did not show any associations in the STRING analysis (see the bottom of Figure 7).

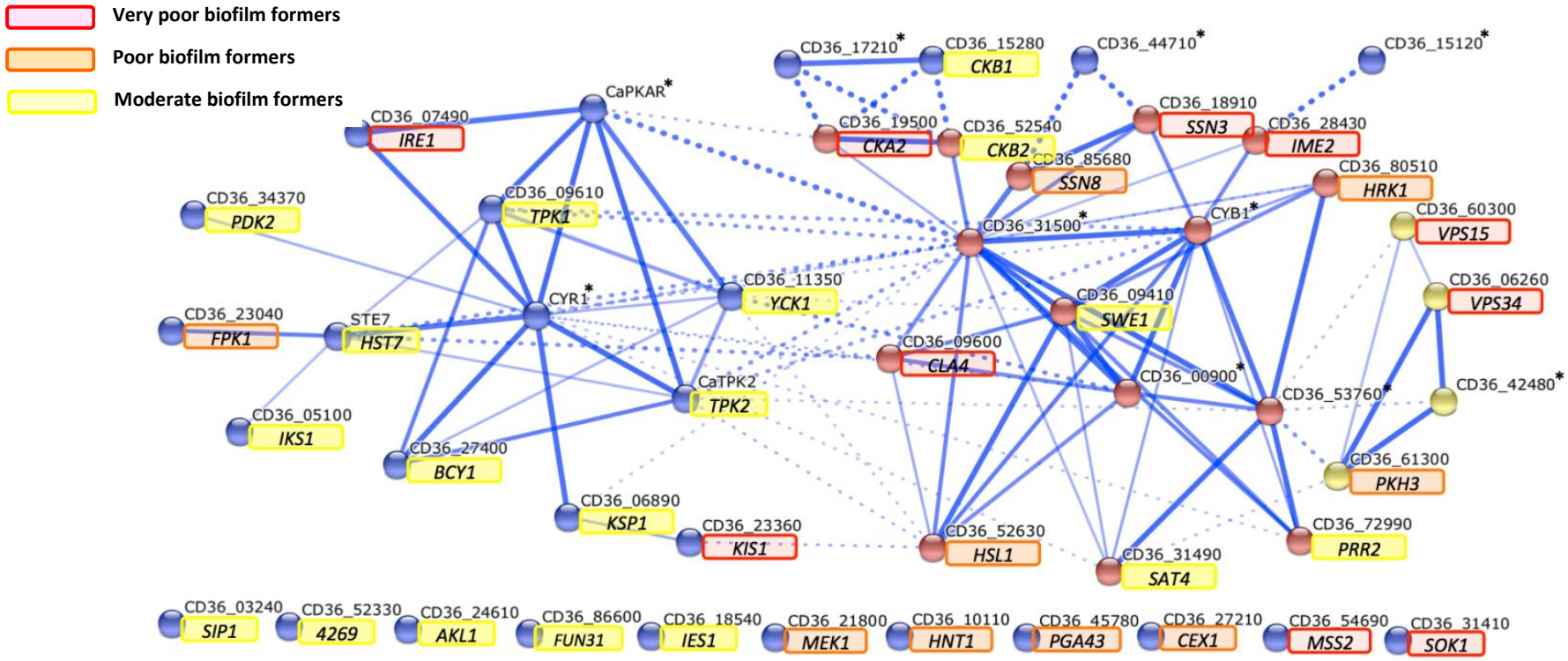


Figure 7| **Predicted STRING interaction networks.** In order to produce an interaction network, the STRING database quantitatively integrates experimental data, computational prediction methods and published literature. The spheres and edges represent PKs and known/predicted interactions respectively. Stronger blue lines indicate the connections with higher confidence. Protein kinase encoding genes are boxed and the asterisk (*) indicates a functional partner predicted by the STRING database. A network shows the interaction of the 37 biofilm-related proteins/genes and their functional partners (*SKY2* was not present in the STRING database).

Table 4| KEGG pathways from the STRING database.

Analysis of the impaired biofilm former mutants	
Pathway	**Gene**
Meiosis	*TPK1, TPK2, SWE1, IME2, MEK1*
PKA	*TPK1, TPK2, BCY1*
Cell cycle	*SWE1, HSL1*
Ribosome biogenesis	*CKB1, CKB2, CKA2*
Regulation of autophagy	*VPS15, VPS34*
Protein processing in ER	*IRE1*
Inositol-phosphate metabolism	*VPS34*
Phagosome	*VPS34*
Metabolic pathways	*VPS34*
Phosphatidylinositol signaling	*VPS34*

Table 5| Functional partners predicted by the STRING database.

C. albicans ortholog	***C. dubliniensis***	**Description**	**Length**
Functional partners of the impaired biofilm former mutants			(aa)
BCY1 (C2_01110C)	*CaPKAR*	PKA regulatory subunit	458
CDC5 (C1_00950C)	CD36_00900	Cell cycle serine/threonine-protein kinase (putative)	653
CDC28 (CR_06050W)	CD36_31500	Cdc28 homologue (putative)	317
CLB2 (C2_01410C)	*CYB1*	G2/mitotic-specific cyclin (putative)	486
CYR1 (C7_03070C)	*CYR1*	Adenylate cyclase (putative)	1690
MEC1 (C5_04060C)	CD36_53760	DNA-damage checkpoint kinase, ATR homologue (putative)	2326
NDT80 (C2_00140W)	CD36_15120	Meiosis-specific transcription factor (putative)	509
POB3 (C2_02380W)	CD36_17210	DNA polymerase delta binding protein	538
SRB8 (C4_05090C)	CD36_44710	Subunit of the RNA polymerase II mediator complex (putative)	1755
C4_02670W	CD36_42480	Autophagy-related protein (putative)	519

2.4 Discussion

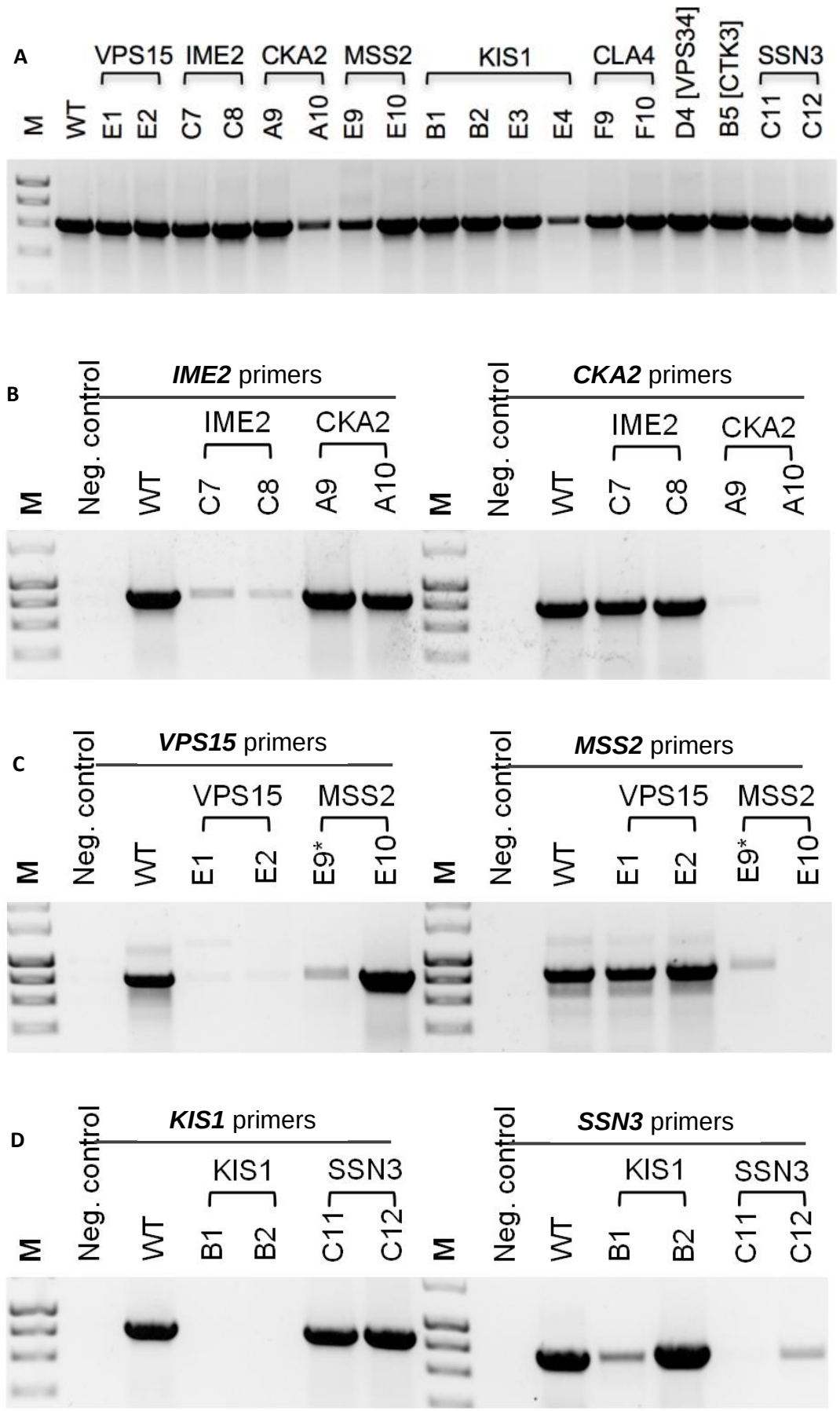
Biofilm formation on the surface of catheters is a leading cause of *C. albicans* infections and drug resistance in hospitals. The aim of our study was to establish whether particular PKs were essential for biofilm formation in *C. albicans* as this might reveal novel processes and pathways that could serve as drug targets. We identified 38 PK mutants that showed some degree of biofilm impairment, but some of them were already linked with biofilms. Because there is an established link between filamentation and biofilm formation, we assessed the capacity of the 20 mutants that were severely impaired in biofilms to switch from yeast to hyphal growth and found a complete coincidence of switching and biofilm formation. Thus, although processes other than filamentation can be required for biofilm formation, either our assay was biased for mutants with filamentation defects or none of the protein kinases in our screens is involved in these other processes. There were, however, some novel findings and five strains were mutated in genes (*VPS15, PKH3, PGA43, IME2* and *CEX1*) not previously directly associated with either filamentation or biofilm formation. It is important to note that other studies used different types of filamentation/biofilm assays (and media), which may explain minor variance between studies.

VPS15 encodes a kinase involved in vacuolar protein sorting and although this gene was not previously linked to biofilms, other members of the Vps family, Vps1p and Vps34p (also identified in our screens), were reported to significantly contribute in filamentation and biofilm formation in *Candida* (Bernardo *et al*. 2008). Furthermore, the *S. cerevisiae vps15* mutant also forms impaired biofilms (Vandenbosch *et al*. 2013). Vps15p and Vps34p form a complex on the vacuole/Golgi membrane, thus these data indicate that correct protein sorting is required for proper fungal development, though this is likely to be an indirect rather than a direct effect. *PKH3,* which encodes a kinase that may be involved in Protein Kinase C (PKC) activity*,* was not previously described as being involved in morphological development but the mutant is reported to display significantly decreased cell-substrate adherence (Fanning *et al*. 2012), an important early step for biofilm development (Finkel and Mitchell 2011). Interestingly, the network analysis showed that Pkh3p is associated with the vacuolar proteins Vps15p and Vps4p, perhaps indicating that the defect in this mutant is also related to protein sorting in the vacuole. *PGA43* encodes a Glycosyl-Phosphatidyl-Inositol (GPI)-anchored protein and thus is likely to be present at the cell surface. Its function is not known but other also Gpi family proteins, for example Pga59p and Pga62p, are required for cell wall integrity (Moreno-Ruiz *et al*. 2009). There is very

limited information available about *C. albicans IME2* but in *S. cerevisiae IME2* is required for pseudohyphal growth independently of the MAPK filamentation cascade (Strudwich *et al*. 2010). Thus, its involvement in morphological development in *C. albicans* should not be surprising. Cex1p (in *S. cerevisiae*) binds to tRNA for its export from the nucleus (McGuire and Mangroo 2007) and has not previously been linked to morphology.

Apart from vacuolar process, our analysis also showed that the PKA pathway, ribosome biogenesis and some aspects of the cell cycle are required for efficient filamentation and biofilm development. The mutants disrupted in *C. albicans* PKA genes, *BCY1*, *TPK1* and *TPK2* formed moderate biofilms. Bcy1p is a regulatory subunit of the PKA and, Tpk1p and Tpk2p are well-documented to be involved in *C. albicans* biofilm formation and filamentation. *CKA2, CKB1* and *CKB2*, genes required for ribosome biogenesis processes, also exhibited impaired biofilms under our assay conditions. Elevated transcription of ribosome biogenesis genes of biofilm forming cells was previously reported by Garcia-Sanchez *et al*. (2004) and Bonhomme *et al*. (2011). The next step now would be to indentify the Achilles heel of the biofilms based on several most important elements of these processes.

2.5 Appendix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CLA4
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C7
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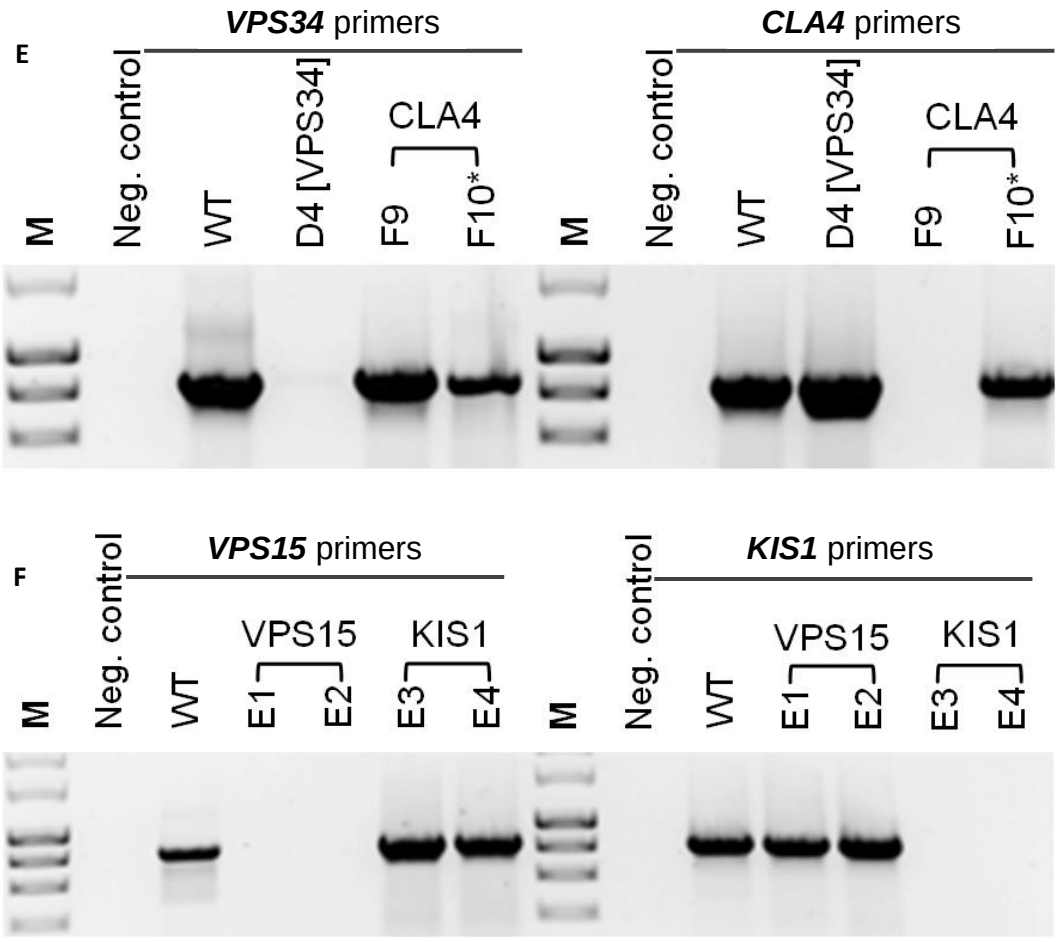

Figure 8| **Examples of molecular validation of the 'Very poor' biofilm former mutants with strain-specific PCR. A**| DNA quality control using the NL4 (GGTCCGTGTTTCAAGACGG) and NL1 (GCATATCAATAAGCGGAGGAAAAG) oligonucleotides as the forward and reverse primers respectively. The pair of NL4 and NL1 primers target the variable 28S ribosomal DNA. Estimated fragment size equals to ~680 bp. **B**| **C**| **D**| **E**| **F**| Cross validation of *C. albicans* PK mutants with the primers for a disrupted gene and any randomly chosen set of primers. Estimated fragment sizes equal to ~820 bp (B, C, D, E and F). DNA isolated from each mutant is indicated on the top of each column and the primers amplifying a particular gene are written in bold italics. Hyperladder I was used as a marker and *C. albicans* DAY286 as a wild-type (WT) control. *Controversial mutant DNA.

Chapter 3

Communication between *Candida albicans* and *Pseudomonas aeruginosa*

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Key words: *Candida albicans*; protein kinase; biofilms; filamentation; yeast - hyphae; *Pseudomonas aeruginosa*.

***Subtitle**: Candida albicans morphology and interaction with Pseudomonas aerugionsa*

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Abstract

C. albicans biofilm development is a complex phenomenon that mainly implicates processes such as adhesion, filamentation supported by intensive cell wall biosynthesis, excretion of the extracellular matrix (ECM) and dispersal of blastospores. The filamentation is largely governed by the MAPK pathways but our analysis of the 'Good' biofilm former PK mutants showed that the individual components of these pathways are not required for robust biofilm formation, indicating functional redundancy. The 'Good' biofilm formers were established after the analyses of their biofilms with a biofilm assay (XTT) and with the assessment of their clusters in the STRING networks. These mutants were further investigated for their response to bacterial supernatants, since *C. albicans* biofilm impairment with *P. aeruginosa* supernatants, was well-documented by the previous publications of McAlester *et al*. (2008) and Holcombe *et al*. (2010). These supernatants contained QS (quorum sensing) molecules derived from 2 distinct strains of *P. aeruginosa* the wild-type, PAO1, and a homoserine lactone-free (HSL) mutant, ΔQS. The biofilm assay showed that *P. aeruginosa* supernatants inhibited biofilm formation in all mutants, regardless of the presence of HSLs. To clarify an effect of the HSLs on *Candida* morphology and filamentation we conducted a morphology assay. This assay suggested that the morphology of *Candida* was affected only by the HSL-containing supernatants that were derived from the wild-type *P. aeruginosa*, PAO1. This confirms the distinct HSL-dependent inhibition of filamentation and the HSL-independent impairment of biofilm development by *P. aeruginosa* supernatants.

3. Communication between *Candida albicans* and *Pseudomonas aeruginosa*

3.1 Introduction

Apart from the established environmental factors, *C. albicans* morphology and biofilm formation is also influenced by some environmental microorganisms such as the Gram-negative bacterium *P. aeruginosa* (reviewed by Peleg *et al*. 2010 and Holcombe *et al*. 2011). *P. aeruginosa* is an opportunistic human pathogen capable of causing inflammation and sepsis by colonizing kidneys, urinary and gastrointestinal tract of the susceptible individuals. Clinical observations suggest that *C. albicans* and *P. aeruginosa* populations can coexist and influence each other (Bauernfeind *et al*. 1987; Kerr 1994; Nseir *et al*. 2007; Chotirmall *et al*. 2010). Notably, *Candida* and *Pseudomonas* were coisolated from the lungs of Cystic Fibrosis (CF) patients (Martin *et al*. 1993; Leclair and Hogan 2010), serious burn wounds (Gupta *et al*. 2005) and the urinary catheters (Falleiros de Pádua *et al*. 2008). *In vitro* analysis established that *C. albicans* biofilm formation and metabolism can be influenced by *P. aeruginosa* phenazines (Gibson *et al*. 2009; Morales *et al*. 2013). For example, *P. aeruginosa* phenazines enhance *C. albicans* ethanol production which stimulates *Pseudomonas* biofilm formation (Chen *et al*. 2014). Another phenazine, methosulphate (PMS), can kill *Candida* within its biofilms (Morales *et al*. 2010; Morales *et al*. 2013). Several studies have also shown that *Pseudomonas* can inhibit *Candida* biofilm development *in vitro* (Holcombe *et al*. 2010; Bandara *et al*. 2010a; Bandara *et al*. 2010b; Reen *et al*. 2011; Bandara *et al*. 2013). These studies collectively suggest that *Candida* biofilm development and metabolism are closely related (Lindsay *et al.* 2014).

Communication between *C. albicans* and *P. aeruginosa* is based on signalling. *P. aeruginosa* can inhibit the *C. albicans* morphological switch from yeast to hyphae using secreted chemicals. Several studies suggest that *Pseudomonas* QS (quorum sensing) molecules are responsible for the signal-mediated communication between the two species (Hogan and Kolter 2002; McAlester *et al*. 2008; Deveau and Hogan 2011). It was reported that HSLs can inhibit the switch of *Candida* from yeast to filamentous growth (Hogan *et al*. 2004). However, *C. albicans* QS molecule, farnesol, can limit the virulence of *P. aeruginosa* by blocking the production of *Pseudomonas* QS molecules and pyocyanin (Cugini *et al*. 2007) and affecting motility (McAlester *et al*. 2008). *P. aeruginosa* pyocyanin and 1-hydroxyphenazine can prevent the growth of *C. albicans* (Kerr *et al*. 1999). We further

investigated whether deletion of the PK-encoding genes disrupted in the 'Good' biofilm former mutants affected the signal-mediated interaction between *P. aeruginosa* and *C. albicans*.

3.2 Materials and Methods

3.2.1 Yeast strains and growth conditions

The wild-type *C. albicans* strains used in this study were SC5314 (Gillum *et al*. 1984), BWP17 (*ura3*Δ::λ*imm434/ura3*Δ::λ*imm434 his1*::*hisG/his1*::*hisG arg4*::*hisG/arg4*::*hisG*) (Wilson *et al*. 1999) and DAY286 (*ura3::λimm434 his1::hisG pARG4::URA3::arg4::hisG*) (Davis *et al.* 2002). The PK mutants of *C. albicans* are listed in Table 1 (Chapter 2). This kinase set was created by Aaron Mitchell's group (Blankenship *et al*. 2010) and obtained from the Fungal Genetics Stock Centre (www.fgsc.net/candida/FGSCcandida resources.htm). PK homozygous insertion mutants were created in BWP17 parental strain via Tn7-UAU1 cassette (Blankenship *et al*. 2010). The CGD descriptions of the *C. albicans* PK mutants can be found in the Appendix 3.5 (Table 5). The majority of the PK-coding genes were represented by double independent mutant strains but in some cases only a single mutant was available. In total, we examined 63 genes using 45 independent duplicate mutants and 18 single mutants. The initial FGSC collection was larger and included mutants deleted in ~80 PK/PK-related genes. Our reduced set comprised the mutants that, after delivery and culturing, we were able to verify by strain-specific PCR using primers flanking the gene insertion sites (see PCR below).

Yeast strains were routinely cultured in standard rich medium containing 1% yeast extract, 2% peptone and 2% glucose (YPD). For biofilm and morphological analyses, the yeast strains were pre-grown in non-filament-inducing medium YNB (yeast nitrogen base), as described by McAlester *et al*. (2008) and Holcombe *et al*. (2010) with some adjustments. Briefly, filter-sterilized YNB salts without amino acids (Difco 291940) were supplemented with 0.2% glucose, 0.1% maltose and 0.16% filter-sterilized synthetic amino acid drop-out Leu^- (Kaiser Formedium DSCK052). For the induction of hyphal growth the strains were transferred to filament-inducing medium, YNBNP, which consisted from YNB supplemented with 2.5 mM *N*-acetyl-D-glucosamine (Sigma A8625) and 25 mM phosphate (sodium) buffer (pH 7).

3.2.2 PCR

DNA of wild-type *C. albicans* and PK mutants was extracted according to Hoffman (2001) protocol. The primers were designed with SnapGene (www.snapgene.com) and evaluated via Primer-BLAST (www.ncbi.nlm.nih.gov). Strain-specific PCR was carried out using primers flanking the gene insertion sites listed in the Supplementary Table S1 of Blankenship *et al*. (2010) publication. Absence of the band indicated a mutant disrupted in the gene amplified

by the corresponding primers. The primer sequences are listed in the Table 1. All the PCR reactions were carried out utilising a GoTaq Green Master Mix (Promega) according to the manufacturers' instructions.

3.2.3 Bacterial strains and preparation of supernatants

The *P. aeruginosa* strains used in this study were a wild-type PAO1 (Holloway and Morgan 1986) and a HSL-negative mutant, ΔQS (Δ*lasRI*::Gm Δ*rhlRI*::Tc) (Beatson *et al*. 2002). *Pseudomonas* supernatants were prepared as described by McAlester *et al*. (2008). Briefly, after overnight growth of *Pseudomonas* in LB broth (37^{o}C) the culture was inoculated into 100 mL of fresh LB and further grown until it reached an approximate absorbance of A_{600}1.2. To harvest the cells, the cultures were centrifuged twice (15 min, 5000 rpm). The resulting supernatants were filter-sterilized with 0.2 µm pore size filter, lyophilized and used immediately or stored at –80^{o}C. Before utilization, the supernatants were re-suspended to yield concentration of 2x from a 20x stock in sterile dH_2O. Sterile LB broth was used as a control after it was prepared in the same manner as the above supernatants.

3.2.4 Biofilm assay

A microtiter plate-based biofilm assay (XTT reductase salts) was carried out as described by Ramage *et al*. (2001) and Holcombe *et al*. (2010). Briefly, the yeast strains were pre-grown in non-filament-inducing medium (YNB) overnight (30^{o}C) and diluted into filament-inducing medium (YNBNP) to A_{600}0.05. The diluted cultures (100 µL) were incubated for 1 h (37°C) in flat-bottomed 96-well polystyrene plates. After incubation, the attached cells were washed with fresh YNBNP medium twice, by inverting the plates carefully, to eliminate non-adhered cells. Where indicated, YNBNP (100 µL) with the *Pseudomonas* supernatant (2x) and LB (2x) was added to the sample and the control cultures respectively. In order to induce biofilm formation the plates were incubated in the dark statically for 24 h (37°C). The next day, the biofilms developed on the bottom of the plates were washed with fresh YNBNP by careful pipetting. The XTT-menadione solution (100 µL), prepared as below, was added to the overnight cultures and incubated in the dark for 2 h (37^{o}C). Lastly, the dyed supernatants (80 µL) were transferred to a clean plate for the quantification at a wavelength of A_{490} nm.

Metabolic activity of the cells was measured with a XTT (reductase salts) assay. For the preparation of the XTT solutions, 10 mM menadione (Sigma M5625) was dissolved in pure acetone and added (10 µL) to the XTT solution. The latter was prepared by dissolving 0.01 g of XTT powder (Sigma X4626) in 20 mL sterile dH_2O and filtered with 0.2 µm pore size filter.

To increase the statistical power of our experiments we tested the mutants in triplicate with eight technical replicates (n = 24). Biofilms of the control, wild-type *C. albicans* DAY286, ranged from approximately $A_{490}1$ to $A_{490}1.5$. Thus, the mutants with biofilms above $A_{490}1$ and below $A_{490}0.5$ were assigned to the 'Good' and 'Poor' biofilm formers respectively. The remaining mutants were 'Moderate' biofilm formers. A distribution of each sample was assessed and the coincidence of the median and the mean indicated a Gaussian (normal) distribution of our data. All the statistical analysis of the biofilm assays were carried out in R Statistical Software (R Development Core Team 2013).

3.2.5 Morphological analyses

Morphological assays were carried out as described previously by Hogan *et al*. (2004) and McAlester *et al*. (2008). Briefly, the yeast cultures were pre-grown overnight in YNB broth (30^{o}C) and diluted into YNBNP ($A_{600}1.0$) to assay their capacity to switch from yeast to filamentous growth. Before (0 h) and after 6 h and 10 h incubation (37^{o}C), the morphology of the mutants was examined microscopically. Where indicated, before incubation for 6 h (37^{o}C), the sample and the control cultures were treated with the bacterial supernatants (2x) and LB (2x) respectively. Since the previous experiments of McAlester *et al*. (2008) and Holcombe *et al*. (2010) had shown that the morphology of *C. albicans* SC5314 was impaired by the *Pseudomona*s supernatants, SC5314 was used as an additional control. The images were captured digitally at a magnification x 400 via Zen2008 software with EX405 BP and Channel D by Zeiss Laser Scanning Microscope (LSM) 510.

3.2.6 Bioinformatics analyses

The protein sequences of *C. albicans* PKs were retrieved from the CGD (*Candida* Genome Database) (Arnaud *et al*. 2005), the analysis of the protein associations were carried out using the STRING v9.1 database (Search Tool for the Retrieval of Interacting Genes/Proteins) (Jensen *et al*. 2009), the GOs (Gene Ontologies) were obtained with the Blast2GO annotations (Conesa *et al*. 2005) and pathway analysis were carried out using KEGG pathway database (Kyoto Encyclopaedia of Genes and Genomes) (Kanehisa and Goto 2000).

Table 1| Primers used in this study.

Forward	Sequence (3' – 5')	Reverse	Sequence (3' – 5')
ATG1_F	CGTTGAGTTTGTTGGGAAGAGCC	ATG1_R	CCTTAAGTGCAGTTTCCCTAGCG
BCK1_F	GAAACTGCAAAGCACAGATACTTTACAGC	BCK1_R	GAGCAACTCTCCTGCAGTGGG
BUB1_F	CGATCGTGTGCTAAGCTCGC	BUB1_R	AACTCTTGACCACAATAGTCGATTATGGC
CEK2_F	ATGAAGAAATCTACTGGCCCC	CEK2_R	TTACGACATGACTATTTCGAAAATTTGTT
CHK1_F	CCCCAAGGTTTTGACAATAGCCG	CHK1_R	GAGAATGTACCCTGGTATCGAATCGG
CPP1_F	CACCACTATCGAGTTATTCAACTACCG	CPP1_R	GTGGTTGTGGAAATACTCGTGGG
CST20_F	GATACAAGTGAAAACCCTGATGACGC	CST20_R	CTTTGTTGGCATTGACTGAGATGGG
DUN1_F	CTTGCACTTGTGTCTTTAGAAATTGAAGG	DUN1_R	TATCATGTGCTGGTGTATGTTGGG
GCN2_F	CAATTTTACATGTGAAGTGGAACGGG	GCN2_R	CCACCATTCAAAGATGAGAATGAATTCCC
HNT1_F	GGCTTCTCATGCTTCCTGTATATTCTG	HNT1_R	CAACAACTTGATGAGCAATTCTTCCG
HOG1_F	ATGTCTGCAGATGGAGAATTTACAAGAAC	HOG1_R	TTAAGCTCCGTTGGCGGAAT
HST7_F	CCATCATCGCCAGCATTATCAAAATAGCC	HST7_R	GGAGACTGCGATGAAGCTGGC
MEC1_F	CATTCCATTGGATTGGTCATTGATGGG	MEC1_R	CCGGATTCTTCATAACAGTGTTGGC
MEK1_F	CAGTGCACCTGAAGTATTCAAGGC	MEK1_R	CTCAGAATAGAAAGGCGAAACACCG
MKC1_F	CAAGTCGTTTGAGACTGTAGATGGG	MKC1_R	CTTTCTCTTCCTCCTCTTGTCGC
MKK2_F	GCCCCAGAACGTATCACTGGG	MKK2_R	CATTAGCCACTTCCAAAAGACTCATACCC
orf19.3720_F	CATCGCTACGACATGACATAACCG	orf19.3720_R	TTGGGGCATTCTTGAACAATGGG
PBS2_F	CTGAAAGTCTTACATAAACCCACCGG	PBS2_R	GGTCTCATAGTGTTGATCCTTTCCG
PKC1_2056_F	CTGACCTTAGTTTTGAAACTGGTTACGG	PKC1_2056_R	GAACACCTTGTATTTGATCAGCAAAGGG
PKC1_3114_F	GCATATGATAGAAGTGTTGATTGGTGGG	PKC1_3114_R	GCATTGTCAGATATATGCGAGAACCC
PRR1_F	CCACCAACATTCAGACTGATTTCCC	PRR1_R	GCACAGATATAATTTTCACAGCAACTGCC
RCK2_F	GTAGGTTGTTGACTGTGGACCC	RCK2_R	GAGCAACTTTTGTAGCTGGTGGG
SKY1_F	GGGTCATTTTTCTACCGTGTGGC	SKY1_R	CTTCTTCCACTTCTACCTAATGAAGGGG
SLN1_F	GATGAAAACTGCATTAGCATCAAATTCCG	SLN1_R	TCTGTGGCCTCTTTTAATTTTGTAATCGG
STE11_F	CAAATCCCGTTAGTCAATGAGAATGGG	STE11_R	CATTAGTACACCATTCAGGAATTTGCGG
YCK2_F	GCATCTTAATGGTGGTAGAGGTTGGG	YCK2_R	CATTTGGTGGATTAGGATGTCCATAACCG

3.3 Results

3.3.1 STRING network analyses

After establishment of the processes important for biofilm development described in Chapter 2, it was interesting to identify the processes not absolutely required for biofilm formation by examining the interactions among the 25 PKs that were dispensable for good biofilm formation (Table 3, Chapter 2). These PKs were analysed for their interactions using STRING database. Figure 1 shows the densely connected network that confidently associated the elements of the MAPK signalling pathway, *STE11*, *CPP1*, *BCK1* and *MKK2* with a hub *HOG1*, an osmotic, heavy metal and core stress response kinase (Figure 1). A smaller cluster showed the interactions of three proteins involved in the cell cycle checkpoint (Dun1, Bub1 and Mec1). The proteins of the clusters mostly shared similarity in function or occurrence in the same pathway. The 25 proteins were also analysed for their function in the KEGG annotated pathways (Table 2). A list of functional partners determined by the STRING database can be found in the Table 3.

Table 2| KEGG pathways from the STRING database.

Analysis of the good biofilm former mutants	
Pathway	**Gene**
MAPK signalling	*HOG1, CPP1, BCK1, STE11, MKK2*
Meiosis	*BUB1, MEC1, SPS1, RIM15*
Cell cycle	*BUB1, MEC1, DUN1*
Protein processing in ER	*GCN2*
Ribosome biogenesis	*CKA1*

Table 3| Functional partners predicted by the STRING database.

C. albicans ortholog	***C. dubliniensis***	**Description**	**Length**
Functional partners of the good biofilm former mutants			
BUB3 (C5_03240W)	CD36_52990	Cell cycle arrest protein (putative)	373
CDC20 (C6_01150W)	CD36_61260	APC/C activator protein (putative)	702
PBS2 (C3_06070C)	CD36_86000	MAP kinase kinase (putative)	536
RAD9 (C5_02610C)	CD36_52380	DNA repair protein Rad9 homologue (putative)	1065
RAD53 (C3_03810W)	CD36_83790	Serine/threonine-protein kinase Rad53 homologue (putative)	700
RFA1 (C2_00380C)	CD36_15350	Replication factor-A protein 1 (putative)	623
RFA2 (C2_07120W)	CD36_21400	Replication factor A protein 2 (putative)	268
SUI2 (C1_06960W)	CD36_06510	eIF-2-alpha (putative)	300
TEL1 (C6_03010W)	CD36_63010	DNA-damage checkpoint kinase (putative)	2873
C1_11370C	CD36_10660	Hypothetical protein	730

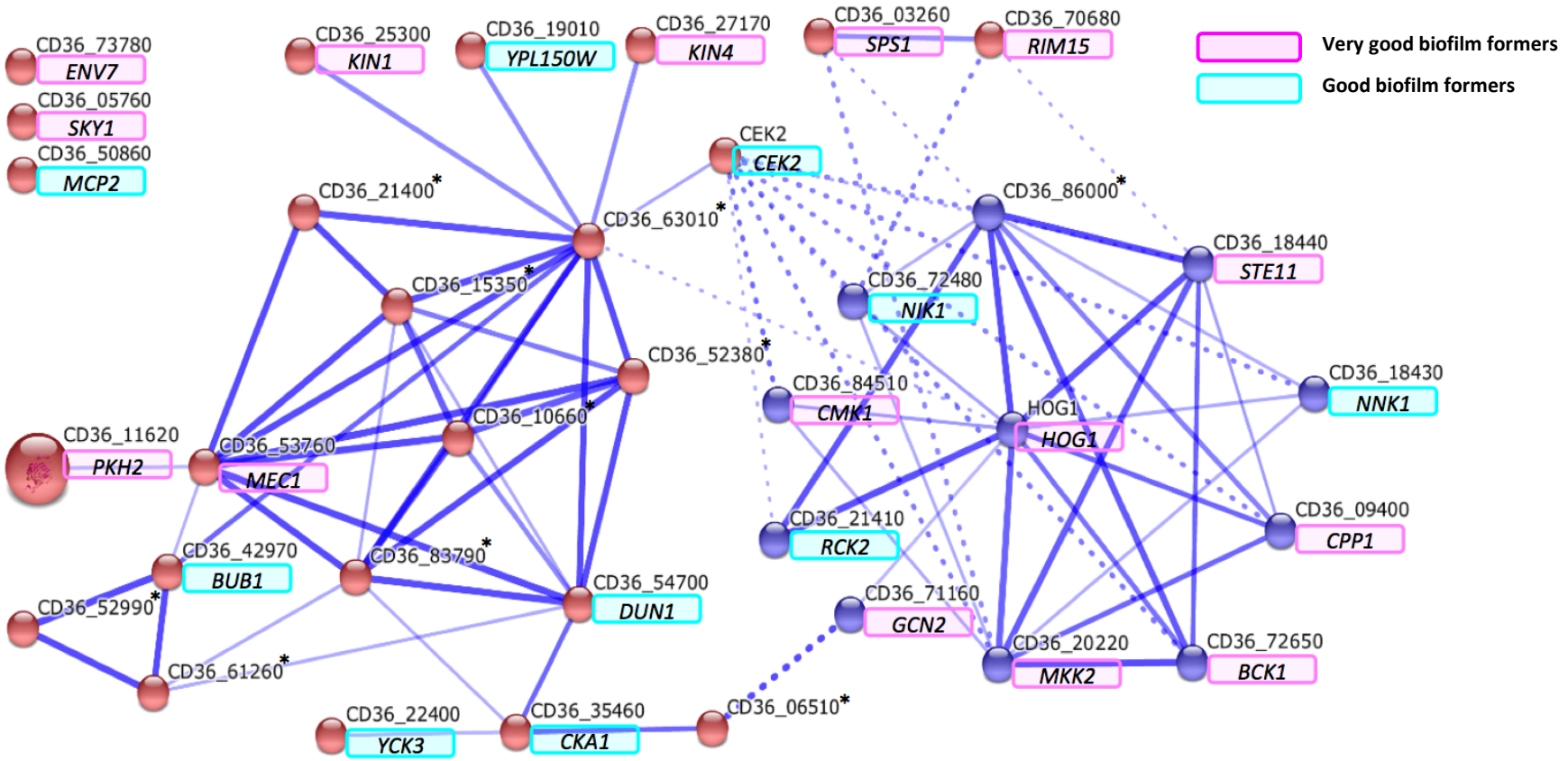


Figure 1| **Predicted STRING interaction networks.** In order to produce an interaction network, the STRING database quantitatively integrates experimental data, computational prediction methods and published literature. The spheres and edges represent PKs and known/predicted interactions respectively. Stronger blue lines indicate the connections with higher confidence. Protein kinase encoding genes are boxed and the asterisk (*) indicates a functional partner predicted by the STRING database. A network shows the interaction of the genes/proteins not individually required for biofilm formation.

3.3.2 Bacterial supernatants inhibit biofilm formation of the 'Good' biofilm formers

Several groups have reported that *P. aeruginosa* secreted signals negatively impact on *C. albicans* morphology and biofilm development. In some cases, this may involve PKs or other proteins acting as signal transducers. Therefore, it was questioned how mutants that still formed robust (good) biofilms responded to *Pseudomonas* supernatants. To address this, *Candida* biofilm formation was assessed after application of the supernatants derived from two strains of *P. aeruginosa*, a wild-type PAO1 and a HSL-free mutant, ΔQS. There was slight difference in how the mutants reacted to the supernatants, with biofilm formation severely inhibited in all cases. However, supernatants derived from the ΔQS strain of *Pseudomonas* (see blue boxplots: s/n DeltaQS) demonstrated less inhibitory effect on the biofilms of mutants disrupted in *STE11*, *BCK1* and *HOG1* genes. This is shown in detail for representative proteins of each major MAPK pathway, *STE11* (regulates growth, mating, cell wall construction and invasive growth), *BCK1* (regulates cell wall integrity, adaption to stress and invasive growth), *CEK2* (participates in mating) and *HOG1* (regulates adaption to stress) (Figure 2).

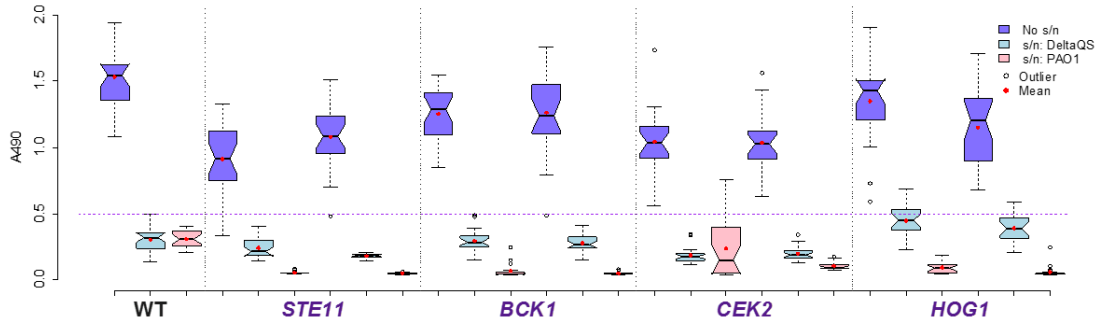
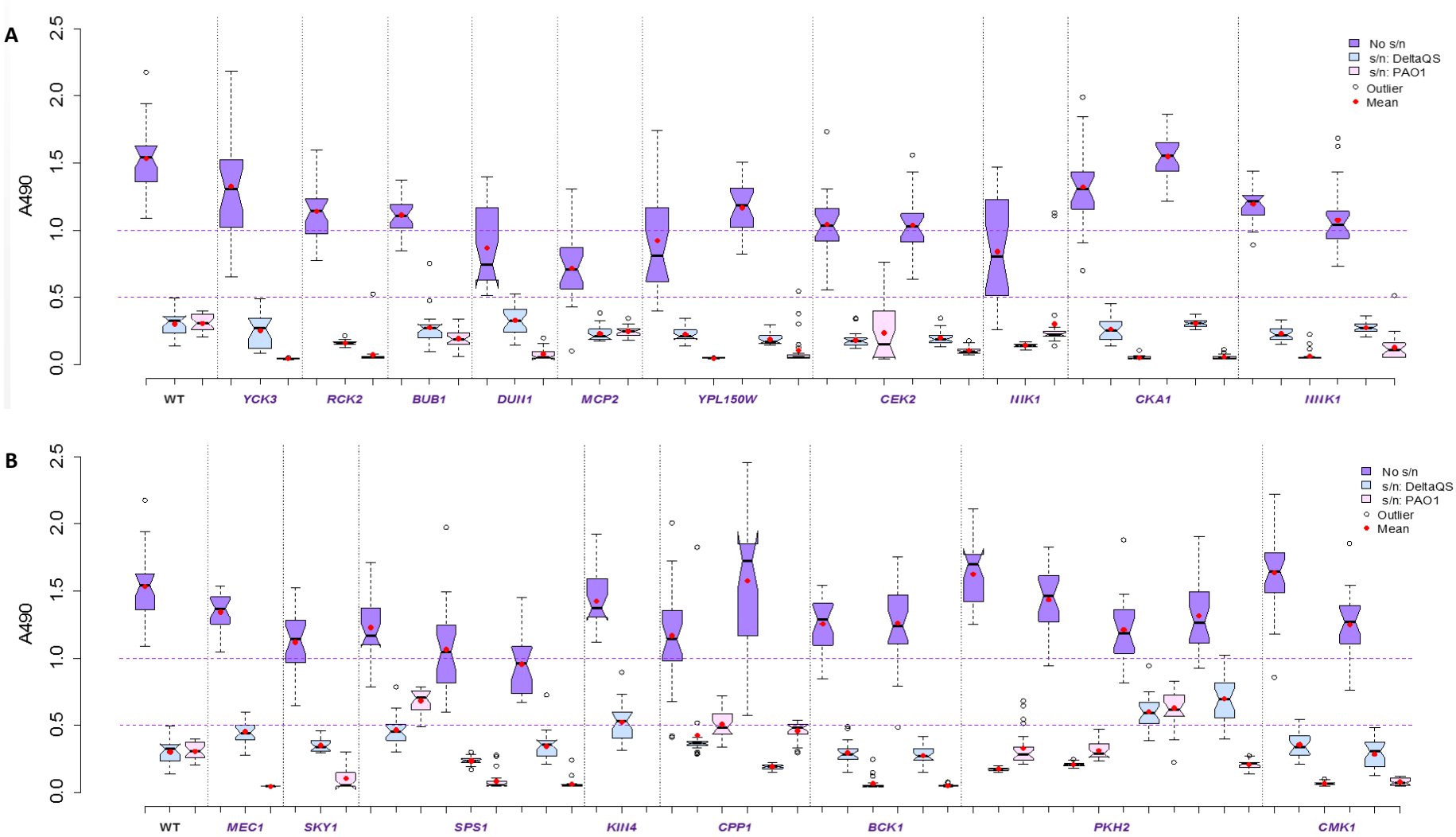


Figure 2| Biofilm formation of representatives of major MAPK pathways in the presence of *P. aeruginosa* supernatants. The effect of *P. aeruginosa* supernatants on the biofilm formation of one representative of four different MAPK pathways was assessed using the standard XTT microtitre plate assay. In each case, two independent mutants were available. The biofilm assay comprised three conditions: control (No s/n); supernatant from a wild-type *Pseudomonas* (s/n PAO1); or supernatant from a mutant *Pseudomonas* lacking HSL molecules (s/n DeltaQS). Wild-type *C. albicans* DAY286 was used as a control. The sample size equals to 24 observations (n = 24). Notched boxplots indicate the distribution of each sample. The notch shows the median confidence interval and the middle line inside the box indicates the median. Red dot indicates a mean, white circle an outlier and a horizontal dashed line shows a threshold $A_{490}0.5$. Data are extracted from Figure 3.

Biofilm formation of all the good biofilm former mutants with and without application of bacterial supernatants is demonstrated in Figure 3. This figure shows that the biofilms of some *C. albicans* PK mutants were less affected by the supernatants derived from a ΔQS strain of *Pseudomonas* lacking HSL coding gene. These mutants are listed in the Table 4.

Table 4| ***C. albicans*** **PK mutants less affected by the ΔQS supernatants**. Data are extracted from Figure 3.

Mutants less affected by the ΔQS supernatants	
'Good' biofilm formers	**'Very good' biofilm formers**
YCK3	*MEC1*
DUN1	*SKY1*
YPL150W	*BCK1*
CKA1	*CMK1*
NNK1	*MKK2*
	RIM15
	ENV7
	STE11
	HOG1


A
A490
0.0
0.5
1.0
1.5
2.0
2.5
No s/n
s/n: DeltaQS
s/n: PAO1
Outlier
Mean
WT
YCK3
RCK2
BUB1
DUN1
MCP2
YPL150W
CEK2
NIK1
CKA1
NIK1
B
A490
0.0
0.5
1.0
1.5
2.0
2.5
No s/n
s/n: DeltaQS
s/n: PAO1
Outlier
Mean
WT
MEC1
SKY1
SPS1
KIN4
CPP1
BCK1
PKH2
CMK1

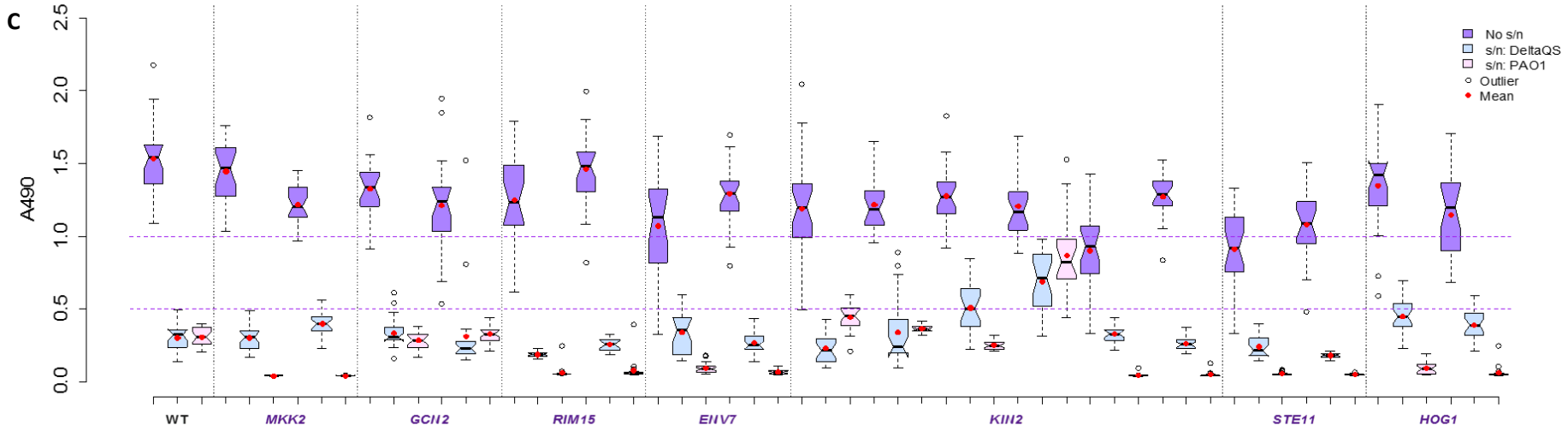


Figure 3| Biofilm formation of the 'Good' biofilm formers after application of the *Pseudomonas* supernatants derived from the wild-type, PAO1, and a mutant, ΔQS. A| Good, **B|** and **C|** Very good biofilm former mutants. The effect of *P. aeruginosa* supernatants on the biofilm formation of mutants was assessed using the standard XTT microtitre plate assay. Wild type *C. albicans* DAY286 was used as a control. The biofilm assay comprised three conditions: control (No s/n); supernatant from a wild-type (s/n: PAO1); or supernatant from a mutant lacking HSL molecules (s/n: Delta QS). Notched boxplots indicate the distribution of each sample. The sample size equals to 24 observations (n = 24). The bottom and the top of the box indicate the 1st and the 3rd quartiles respectively. The notch shows the median confidence interval and the middle line inside the box indicates the median. The upper and the lower limits demonstrate the maximum and the minimum observations respectively. Red dot indicates a mean, white circle an outlier and a horizontal dashed line shows a threshold $A_{490}0.5$ (~1/3 of the wild type biofilm). A coincidence of the mean and median indicates a normal distribution of the data. The statistical analyses were performed in R Statistical Software.

3.3.3 ΔQS bacterial supernatants inhibit biofilm formation of the good biofilm formers without affecting their morphology

Since the biofilms are linked to yeast morphology, we also assessed the mutants' ability to switch from yeast to hyphal growth in the absence or presence of bacterial supernatants (Figure 4). As previously reported by McAlester *et al*. (2008) and Holcombe *et al*. (2010), supernatants from the wild-type *P. aeruginosa* prevented yeast switching to hyphal growth. This was the case for both wild-type *C. albicans* and the mutants tested. In contrast, all the ΔQS supernatant-treated mutants maintained a yeast to hyphae switch (with the possible exception of *CEK2*) consistent with other reports of HSL-dependent and HSL-independent effects.

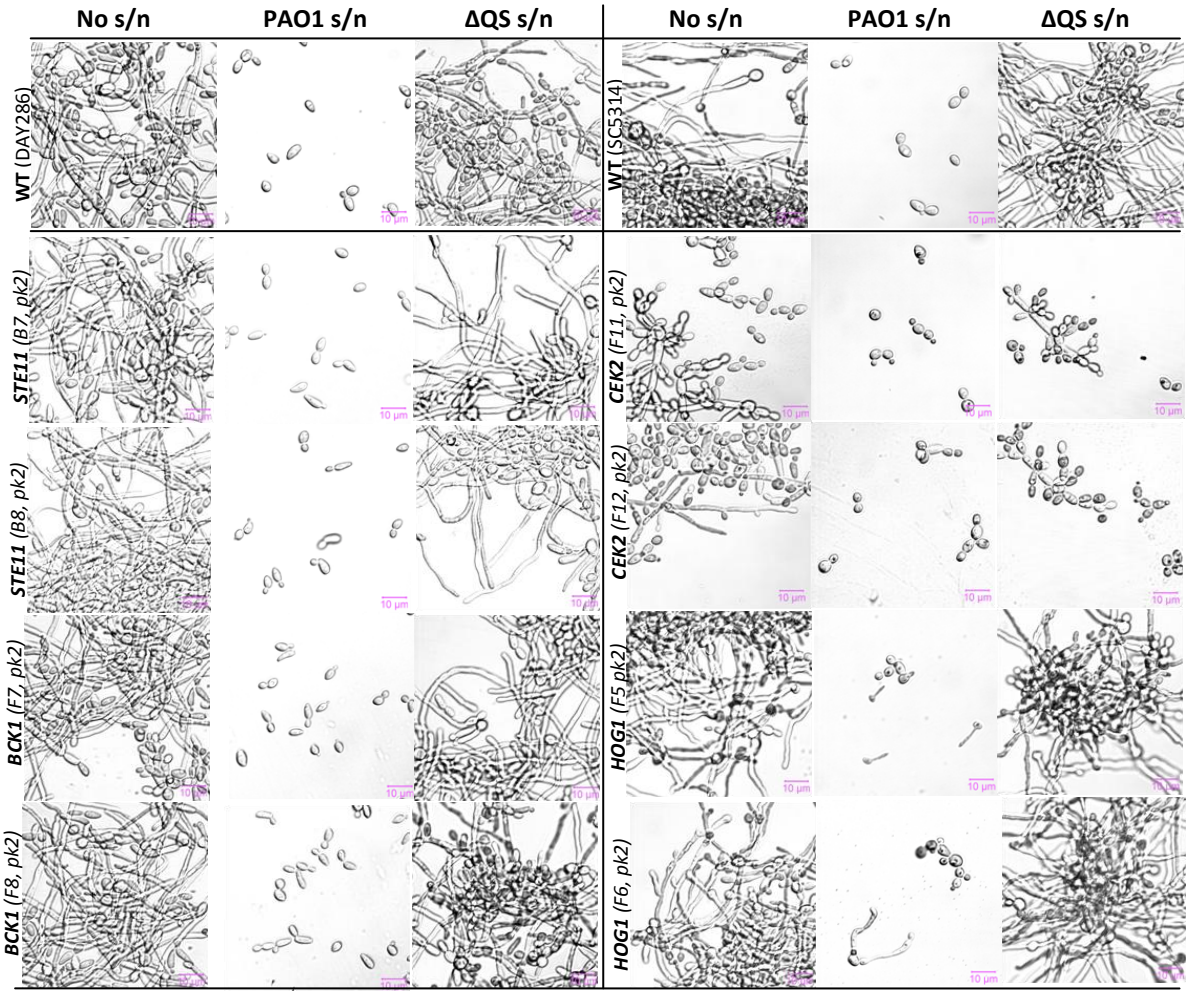


Figure 4| Morphology of representatives of major MAPK pathways in the presence of *P. aeruginosa* supernatants. The effect of *P. aeruginosa* supernatants on morphological development of one representative of four different MAPK pathways was assessed using the standard yeast to hyphae transition assay. Two wild-type *C. albicans* strains, DAY286 and SC5314, and four mutants were tested for filamentation. The morphology of each

strain was examined after 6 h incubation (37^{o}C) in filament-inducing medium (YNBNP). The treatments were: control (No s/n); supernatant from a wild-type *P. aeruginosa* strain (PAO1 s/n); or supernatant from a *P. aeruginosa* mutant lacking HSL molecules (ΔQS s/n). The scale bar corresponds to 10 µm.

3.4 Discussion

Biofilm formation in *C. albicans* is mediated by diverse processes such as adherence and hyphal growth. After highlighting the additional processes that were important for robust biofilm development (Chapter 2), it was also interesting to see which proteins were not absolutely required for biofilm formation and, in this regard, the finding that none of the individual PKs in MAP kinase cascades is required appears surprising. This result can be explained by the redundancy and cross-talk that is built into these pathways and thus is likely that individual mutants are compensated by parallel processes in the cell. Network analysis also suggested that impairing aspects of the cell cycle has no impact on these functions and the presence of cell cycle processes in both the 'Poor' and the 'Good' categories reflects the complexity of these processes in the cell.

Previous studies of McAlester *et al*. (2008) and Holcombe *et al*. (2010) showed that both the yeast-hyphal transition and *Candida* biofilms were impaired by *Pseudomonas* supernatants. In the case of morphology, QS molecules (HSLs) are known to play a role by targeting the Ras/cAMP/PKA pathway (reviewed by Hogan and Sundstrom 2009), whereas quinolones may be involved in the biofilm effect, through an unknown mechanism (Reen *et al*. 2011). We assessed whether any of the mutants that formed good biofilms failed to respond to *P. aeruginosa* supernatants without finding any obvious positives. Furthermore, the remaining PK mutants were also subject to this screen, with the same findings (data not shown). However, mutants were briefly differentiated into HSL-dependent and HSL-independent but none of the protein kinases was strongly related with signal transduction response in *Candida* to *Pseudomonas* signals. Initially, *CEK2* stood out as this mutant failed to filament in the presence of HSL-negative supernatant but close inspection of the control conditions indicated that this is an underlying morphological defect in this mutant (Figure 4). In fact, it is somewhat surprising that the mutant is able to form biofilms given the pseudohyphal nature of the strain (Figure 2 and 4).

The comprehensive analysis of the response of wild-type and 63 mutants to supernatants from wild-type and QS^- (HSL-negative) strains of *P. aeruginosa* unequivocally separates the two effects on *C. albicans*: HSL-dependent impairment of morphology and HSL-independent impairment of biofilms. It remains to be determined whether the latter effect is fully explained by the action of quinolones (natural antimicrobial signalling molecules) or whether it arises from a combination of effects, for examples, quinolones, LPS (lipopolysaccharides) and other molecules.

3.5 Appendix

Table 5|CGD Descriptions of *C. albicans* PK mutants.

ID	Gene	CGD description	Virulence-pathogenicity	Biofilms-filamentation	References
Very poor					
C6_01190C (orf19.130)	*VPS15*	Involved in retrograde endosome-to-Golgi protein transport	Required for normal virulence		(Liu *et al*. 2014)
CR_03290C (orf19.2395)	*IME2*	Ser/Thr PK activity	Hypersensitivity to amphotericin B		(Xu *et al*. 2007)
C2_04980C (orf19.3530)	*CKA2*	Catalytic alpha-subunit activity of protein kinase CK2; synthetically lethal with CKA1	Linked with pathogenesis; attenuated virulence in a mouse oropharyngeal candidiasis but not in a systemic mouse model; interaction with calcineurin pathway affects fluconazole sensitivity		(Nobile *et al*. 2003; Bruno and Mitchell 2005; Chiang *et al*. 2007; Inglis *et al*. 2013)
C5_05090W (orf19.4001)	*MSS2*	Role in protein insertion into mitochondrial membrane from inner side and extrinsic to mitochondrial inner membrane, mitochondrial matrix localization			(CGD 2010)
C2_09230C (orf19.4084)	*KIS1*	Snf1p complex scaffold protein activity; interacts with Snf4p; Hap43p-repressed gene	Mutants are hypersensitive to caspofungin and hydrogen peroxide (H_2O_2)		(Corvey *et al*. 2005; Blankenship *et al*. 2010; Singh *et al*. 2011)
CR_06000W (orf19.451)	*SOK1*	Required for degradation of Nrg1p; induced by alpha pheromone in SpiderM medium	Mutants are sensitive to growth on hydrogen peroxide (H_2O_2) medium	Up in biofilms; rat catheter and Spider biofilm induced	(Bennett and Johnson 2006; Nett *et al*. 2009; Blankenship *et al*. 2010; Nobile *et al*. 2012; Lu *et al*. 2014)
C1_10210C (orf19.4890)	*CLA4*	Ser/Thr PK activity (Ste20p family)	Linked with pathogenesis; required for virulence in mouse systemic infection; mutant caspofungin sensitive	Linked with filamentous growth; required for wild-type filamentous growth and has role in chlamydospore formation	(Martin *et al*. 2005; Leberer *et al*. 1997; Blankenship *et al*. 2010; Inglis *et al*. 2013)
C1_07970C (orf19.5068)	*IRE1*	Role in cell wall regulation	Mutant is hypersensitive to caspofungin	Up in biofilms; spider biofilm induced	(Blankenship *et al*. 2010; Nobile *et al*. 2012)
C1_06680W (orf19.6243)	*VPS34*	Required for normal vesicle transport; autophosphorylated class III phosphatidylinositol 3-kinase activity	Linked with pathogenesis; required for virulence in mouse systemic infection; caspofungin and hydrogen peroxide sensitivity	Linked with filamentous growth; required for hyphal growth and fibroblast adherence and is growth-regulated	(Eck *et al*. 2000; Bruckmann *et al*. 2000; Bruckmann *et al*. 2001; Gunther *et al*. 2005; Blankenship *et al*. 2010; Inglis *et al*. 2013)
C2_04260W (orf19.794)	*SSN3*	Cyclin-dependent PK activity (putative)	Mutants are sensitive to hydrogen peroxide (H_2O_2) medium		(Epp *et al*. 2010; Blankenship *et al*. 2010; Zhang *et al*. 2012)
Poor to moderate					
C6_00350W (orf19.1196)	*PKH3*	Ortholog(s) have protein kinase activity and role in MAPK cascade involved in cell wall biogenesis, protein phosphorylation			(CGD 2010; Pastor-Flores *et al*. 2013)
C2_07530C (orf19.1874)	*MEK1*	Role in meiosis, meiotic recombination checkpoint, protein phosphorylation,			(CGD 2010)

		regulation of linear element assembly and cytosol, linear element localization			
C2_08860W (orf19.223)	*FPK1*	Hap43-repressed; possibly an essential gene, disruptants not obtained by UAU1 method	Induced by prostaglandins	Up in biofilms; flow model biofilm induced; Spider biofilm induced	(Levitin and Whiteway 2007; Mitchell 2009; Bonhomme *et al*. 2011; Singh *et al*. 2011; Nobile *et al*. 2012)
C1_10780C (orf19.2341)	*HNT1*	Similar activity with protein kinase C inhibitor-I; protein level decreases in stationary phase cultures			(Kusch *et al*. 2008)
C4_06260W (orf19.2910)	*PGA43*	Putative GPI-anchored protein activity (posttranslational modification)			(De Groot *et al*. 2003)
C2_06600W (orf19.35)	*SKY2*	Predicted kinase activity		Up in biofilms; rat catheter, flow model, spider biofilm induced	(Nett *et al.* 2009; Bonhomme *et al.* 2011; Nobile *et al*. 2012)
CR_02250C (orf19.3744)	*CEX1*	Ortholog(s) have tRNA binding activity, role in tRNA export from nucleus and cytoplasm, nuclear pore localization			(CGD 2010)
C5_02840C (orf19.4308)	*HSL1*	Involved in determination of morphology during the cell cycle of both yeast-form and hyphal cells via regulation of Swe1p and Cdc28p	Required for full virulence and kidney colonization in mouse systemic infection	Linked with filamentation and biofilms	(Wightman *et al*. 2004; Umeyama *et al*. 2005; Inglis *et al*. 2013)
C3_00550C (orf19.5408)	*HRK1*	Predicted role in cellular ion homeostasis		Down in biofilms; spider biofilm repressed	(Blankenship *et al*. 2010; Nobile *et al*. 2012)
C3_05740C (orf19.7355)	*SSN8*	Ortholog of *S. cerevisiae* Ssn8; a component of RNA polymerase II holoenzyme	Mutants are viable and are sensitive to hydrogen peroxide medium		(Nobile *et al*. 2003; Blankenship *et al*. 2010; Zhang *et al*. 2012)
Moderate					
C7_03340C (orf19.1341)	*PRR2*	Ser/Thr PK activity (putative)	Mutation confers resistance to 5-fluorocytosine (5-FC)		(Xu *et al*. 2007)
C2_00300C (orf19.2102)	*CKB1*	Regulation of casein kinase II (β subunit)	Null mutants are hypersensitive to caspofungin and hydrogen peroxide (H_2O_2) medium		(Zelada *et al.* 2003; Blankenship *et al.* 2010; Bruno and Mitchell 2005)
C2_07210C (orf19.2277)	*TPK2*	Controls morphogenesis and stress response; cAMP-dependent PK catalytic subunit; isoform of Tpk1	Linked with pathogenesis; needed for epithelial cell damage, engulfment and oral (not systemic) virulence in mice	Linked with filamentous growth and biofilms; down in biofilms; rat catheter, spider biofilm repressed	(Sonneborn *et al.* 2000; Bockmuhl *et al.* 2001; Cloutier *et al.* 2003; Park *et al.* 2005; Nett *et al.* 2009; Nobile *et al.* 2012; Inglis *et al*. 2013)
C1_03450C (orf19.3047)	*SIP3*	Protein kinase-related protein activity	Required for normal sensitivity to caspofungin		(Blankenship *et al*. 2010)
CR_02460W (orf19.3720)	*BCY1*	Ortholog(s) have endo-1,4-beta-xylanase activity and SCF ubiquitin ligase complex, cytoplasm localization			(CGD 2010)
CR_06040W (orf19.3854)	*SAT4*	Clade-associated gene expression	Amphotericin B induced	Up in biofilms; spider biofilm induced	(Liu *et al*. 2005; MacCallum *et al*. 2009; Nobile *et al*. 2012)

C5_02560C (orf19.4269)		Protein kinase activity and role in protein phosphorylation; has domain(s) with predicted ATP binding			(CGD 2010)
C1_05370C (orf19.428)	*IKS1*	Induced during planktonic growth; putative serine/threonine kinase activity		Rat catheter biofilm repressed	(Murillo *et al*. 2005; Nett *et al*. 2009)
C5_02760W (orf19.4297)	*CKB2*	Regulation of casein kinase II (β subunit)	Null mutants are hypersensitive to caspofungin		(Zelada *et al.* 2003; Bruno and Mitchell 2005; Blankenship *et al.* 2010)
C1_07380C (orf19.4432)	*KSP1*	mRNA binds She3 and is localized to hyphal tips	Mutation confers hypersensitivity to amphotericin B		(Xu *et al*. 2007; Elson *et al*. 2009)
CR_03900W (orf19.469)	*HST7*	MAPK involved in mating; phosphorylates Cek1p; functional homolog of *S. cerevisiae* Ste7p;	Wild-type virulence in mouse systemic infection; mutants are hypersensitive to caspofungin	Linked with filamentous growth; Involved in hyphal growth signal transduction pathways;	(Clark *et al*. 1995; Kohler and Fink 1996; Leberer *et al*. 1996; Csank *et al*. 1998; Chen *et al*. 2002; Magee *et al*. 2002; Eisman *et al*. 2006; Blankenship *et al*. 2010; Inglis *et al*. 2013)
C1_10010C (orf19.4867)	*SWE1*	Role in control of growth and morphogenesis	Required for full virulence; Linked with pathogenesis; mutant is hypersensitive to caspofungin	Not required for filamentous growth; regulated by 6 biofilm regulators	(Wightman *et al.* 2004; Bennett RJ and Johnson 2006; Gale *et al.* 2009; Blankenship *et al.* 2010; Nobile *et al*. 2012; Inglis *et al*. 2013)
C1_10220C (orf19.4892)	*TPK1*	Controls morphogenesis and stress response; WT nuclear localization requires Bcy1; cAMP-dependent PK catalytic subunit; Tpk2 isoform		Linked with filamentous growth and biofilms; rat catheter and Spider biofilm induced; produced during stationary, not exponential growth	(Bockmuhl *et al.* 2001; Cassola *et al.* 2004; Cloutier *et al.* 2003; Nett *et al.* 2009; Nobile *et al.* 2012; Inglis *et al*. 2013)
C1_12120W (orf19.5253)	*YAK1*	Tyr PK activity and role in protein phosphorylation; domain(s) with ATP binding (predicted)			(CGD 2010)
C2_10750C (orf19.5357)	*AKL1*	Induced during the mating process			(Zhao *et al.* 2005)
CR_08860W (orf19.7281)	*PDK2*	Putative pyruvate dehydrogenase kinase activity	Mutation confers hypersensitivity to amphotericin B		(Xu *et al.* 2007)
C3_06620W (orf19.7451)	*FUN31*	Involved in cell wall damage response; similar to *S. cerevisiae* Psk1p;	Induced by Mnl1 under weak acid stress	Up in biofilms; rat catheter and Spider biofilm induced	(Nobile *et al.* 2003; Rauceo *et al.* 2008; Ramsdale *et al.* 2008; Nett *et al.* 2009; Nobile *et al.* 2012)
C2_03900C (orf19.835)	*IES1*	Ortholog(s) have role in nucleosome mobilization and Ino80 complex, cytosol localization			(CGD 2010)
Moderate to good					
C2_08270C (orf19.2222)	*YCK3*	Plasma membrane-localized			(Cabezon *et al.* 2009)
C2_07130C (orf19.2268)	*RCK2*	Induced by osmotic stress via Hog1p; macrophage / pseudohyphal-repressed	Linked with pathogenesis; mutants are sensitive to rapamycin	Linked with filamentous growth	(Singh *et al*. 2005; Enjalbert *et al*. 2006; Li *et al*. 2008; Inglis *et al*. 2013)
C4_03120C (orf19.2678)	*BUB1*	Checkpoint kinase activity	Mutation confers increased sensitivity to		(Xu *et al.* 2007)

			nocodazole		
C5_05100C (orf19.4002)	*DUN1*	Involved in DNA damage cell-cycle checkpoint; induced under Cdc5p depletion			(Bachewich *et al.* 2005)
C5_01490C (orf19.4144)	*MCP2*	Predicted protein kinase; clade-associated gene expression			(MacCallum *et al.* 2009)
C2_04360W (orf19.4518)	*YPL150W*	Protein kinase of unknown function; mutants are viable			(Nobile *et al.* 2003)
CR_05940W (orf19.460)	*CEK2*	Required for mating; component of the signal transduction pathway that regulates mating	Induced by Cph1, pheromone	Linked with filamentous growth; transposon mutation affects filamentous growth	(Zhou *et al.* 1999; Chen *et al.* 2002; Uhl *et al.* 2003; Dignard and Whiteway 2006; Inglis *et al*. 2013)
C7_02800W (orf19.5181)	*NIK1*	Involved in a two-component signaling pathway that regulates cell wall biosynthesis	Linked with pathogenesis; required for wild-type virulence in mouse systemic infection but not for drug sensitivity / resistance	Linked with filamentous growth; not required for wild-type growth	(Nagahashi *et al.* 1998; Yamada-Okabe *et al.* 1999; Selitrennikoff *et al.* 2001; Buschart *et al.* 2012; Inglis *et al*. 2013)
CR_10660W (orf19.7652)	*CKA1*	Synthetically lethal with CKA2	Cka1p and Cka2p have a common target with respect to fluconazole resistance; flucytosine induced		(Bruno and Mitchell 2005; Liu *et al.* 2005)
C2_03760C (orf19.846)	*NNK1*	Implicated in proteasome function in *S. cerevisiae*	Induced by Mnl1 under weak acid stress		(Ramsdale *et al.* 2008)
Very good					
C5_04060C (orf19.1283)	*MEC1*	Role in genome integrity; RNA abundance regulated by tyrosol and cell density			(Chen *et al*. 2004; Legrand *et al*. 2011)
C1_06090C (orf19.2436)	*SKY1*	Ser/Thr PK activity (predicted)		Up in biofilms; Spider biofilm induced	(Nobile *et al.* 2012)
C1_03470C (orf19.3049)	*SPS1*	Role in activation of bipolar cell growth, ascospore wall assembly, protein phosphorylation and cell division site, cytosol, nucleus, prospore membrane localization			(CGD 2010)
CR_02210W (orf19.3751)	*KIN4*	Possibly an essential gene, disruptants not obtained by UAU1 method			(Mitchell AP 2009)
C1_10000C (orf19.4866)	*CPP1*	Regulates Cst20-Hst7-Cek1-Cph1 filamentation pathway; yeast-enriched	Linked with pathogenesis; required for virulence in mice; induced by alpha pheromone in Spider Mmedium;	Linked with filamentous growth; represses yeast-hyphal switch; Spider biofilm induced	(Csank *et al.* 1997; Csank *et al.* 1998; Cheng *et al.* 2003; Bennett *et al.* 2003; Nobile *et al.* 2012; Inglis *et al*. 2013)
C7_02990W (orf19.5162)	*BCK1*	Role in cell integrity pathway	Mutant is hypersensitive to caspofungin		(Nguyen *et al.* 2004; Monge *et al.* 2006; Blankenship *et al.* 2010)
C1_12410C (orf19.5224)	*PKH2*	Role in sphingolipid-mediated signaling pathway that controls endocytosis (predicted)		mRNA binds She3 and is localized to hyphal tips	(Elson *et al.* 2009; Pastor-Flores *et al.* 2013)
C3_04550C (orf19.5911)	*CMK1*	Expression regulated upon white-opaque switching; biochemically purified Ca2+/CaM-dependent kinase is			(Lan *et al.* 2002; Dhillon *et al.* 2003; Singh *et al.* 2011; Ding *et al*. 2014)

		soluble, cytosolic, monomeric, and serine-autophosphorylated; Hap43p-repressed			
C2_05780C (orf19.6889)	*MKK2*	Involved in signal transduction; macrophage-downregulated	Mutants are hypersensitive to caspofungin		(Nobile *et al.* 2003; Fernandez-Arenas *et al.* 2007; Blankenship *et al.* 2010)
C7_01330C (orf19.6913)	*GCN2*	Nonessential role in amino acid starvation response		Linked with filamentous growth	(Tournu *et al.* 2005; Inglis *et al*. 2013)
C7_00740W (orf19.7044)	*RIM15*	Role in age-dependent response to oxidative stress involved in chronological cell aging, conidiophore development, protein phosphorylation, regulation of meiosis		Role in hyphal growth	(Stichternoth *et al.* 2011)
C7_04110W (orf19.7164)	*ENV7*	Ortholog(s) have role in ascospore formation, conidiophore development, conidium formation, response to oxidative stress, sporocarp development involved in sexual reproduction, vacuolar protein processing			(CGD 2010)
CR_00260W (orf19.7510)	*KIN2*	Transcription is positively regulated by Tbf1			(Hogues *et al.* 2008)
C2_03770C (orf19.844)	*STE11*	Similar activity to *S. cerevisiae* Ste11p	Linked with pathogenesis; mutants are sensitive to growth on hydrogen peroxide (H_2O_2) medium	Linked with filamentous growth	(Lee *et al.* 2005; Bennett and Johnson 2005; Blankenship *et al.* 2010; Inglis *et al*. 2013)
C2_03330C (orf19.895)	*HOG1*	Role in regulation of glycerol, D-arabitol in response to stress; Role in osmotic-, heavy metal-, and core stress response	Linked with pathogenesis; mutant induces protective mouse immune response; phosphorylated in response to hydrogen peroxide (H_2O_2)(Ssk1-dependent) or NaCl	Linked with filamentous growth	(San Jose *et al.* 1996; Alonso-Monge *et al.* 1999; Smith *et al.* 2004; Fernandez-Arenas *et al.* 2004; Kayingo and Wong 2005; Enjalbert *et al.* 2006; Inglis *et al*. 2013)

Chapter 4

TFbsST: Transcription Factor binding site Search Tool

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Key words: *Candida albicans*; transcription factor binding site motifs; biofilms; *Pseudomonas aeruginosa*; promoter regions; TFbsST database.

***Subtitle**: In silico analysis of Candida albicans promoter sequences*

This chapter is in preparation for submission in the Database Issue of Nucleic Acid Research (Oxford Journals). The TFbsST database is published online under the following link: http://bioinfo.ucc.ie/TFbsST/.

Abstract

C. albicans is a serious pathogen that features high drug resistant properties partially due to its biofilms. The biofilm development depends on the environmental stimuli/signals that are sensed by the fungal cell receptors. These signals are transmitted by the protein kinases (PK) and the gene transcription is regulated by the transcription factors (TF). Using *C. albicans* TF binding site library and Python algorithms, we created a TFbsST database (Transcription Factor binding site Search Tool), a user-friendly application for *in silico* analyses of *Candida* promoters. TFbsST database contains TF binding sites of *C. albicans* and related *Candida* species such as *C. parapsilosis, C. dubliniensis* and *C. glabrata*. Additionally, TFbsST performs TF frequency (outputs a table with %) and TF localization analysis (outputs graphs and tables). These features render the TFbsST software as a useful tool for *in silico* analysis of *Candida* gene promoter sequences.

Previous studies of Holcombe *et al*. (2010) had shown that *P. aeruginosa* secreted signals can affect *C. albicans* biofilms. To investigate the regulation of *Candida* genes that were altered in response to *P. aeruginosa* secreted chemicals, we utilised a TFbsST application. The up-to-date gene ontology (GO) annotation indicated that the genes coding for the cell wall/surface proteins, important for biofilm formation, contained members of the biofilm-related gene families, *RBT* and *ALS*. To identify the TFs that were overrepresented in these gene families, we carried out TF frequency analyses. These analyses showed that Efg1p was overrepresented in both *RBT* and *ALS* families. To question whether Efg1p could regulate the *RBT* family genes across other *Candida* species, *C. parapsilosis RBT* family gene promoters were screened against *C. parapsilosis* Efg1p using a Motif search option of the TFbsST application. Efg1p binding sites were predicted to regulate some *C. parapsilosis RBT* genes indicating that Efg1p is an important TF across the different *Candida* species.

4. TFbsST: Transcription Factor binding site Search Tool

4.1 Introduction

A serious human pathogen, *C. albicans*, can cause nosocomial infections partially due to its ability to form drug resistant biofilms on indwelling medical devices. In these clinical settings, *C. albicans* is frequently coisolated with *P. aeruginosa. P. aeruginosa* is the main cause of morbidity in populations with cystic fibrosis (CF) (Govan and Deretic 1996; Chambers *et al*. 2005; Leclair and Hogan 2010) and AIDS (Mendelson *et al*. 1994). Studies from our laboratory (Holcombe *et al*. 2010; Reen *et al*. 2011;) and independent investigations (Bandara *et al*. 2010a; Bandara *et al*. 2010b; Bandara *et al*. 2013) have shown that *Pseudomonas* can inhibit *Candida* biofilm development *in vitro*. *C. albicans* biofilm development is a complex phenomenon orchestrated by more than 1000 genes that are governed by 9 master transcription regulators, Efg1p, Ndt80p, Brg1p, Bcr1p, Rob1p, Tec1p, Flo8p, Gal4p and Rfx2p (Nobile *et al*. 2012; Fox *et al*. 2015).

Transcription factors (TF) regulate expression of target genes during transcription. TFs contain DNA binding domains that facilitate recognition and binding of TFs to specific small DNA sequences across the promoter region (1000 bp upstream) of target genes. A collection of TF DNA binding site data facilitates computational analysis of the gene promoter regions when using appropriate software. A comprehensive TF motif database is useful for *in silico* analysis of transcriptional networks and gene regulation studies. We additionally used our TF binding site database for computational analysis of biofilm regulation in *C. albicans* as well as for the investigation of interaction between *C. albicans* and *P. aeruginosa*.

Given the rapid progress of bioinformatics tools, many databases were developed to analyse the promoter regions of genes in *S. cerevisiae* or other model organisms:

- YeTFaSCo (Yeast Transcription Factor Specificity Compendium) focuses on *S. cerevisiae* TF binding sites that are evaluated for quality using several metrics. These specificities are given in Position Frequency (PFM) or Position Weight Matrix (PWM) formats. YeTFaSCo is mainly used to find the position of potential binding sites of a sequence and for inspection of precomputed genome-wide binding sites (http://yetfasco.ccbr.utoronto.ca) (de Boer and Hughes 2011).

- UniPROBE (Universal PBM Resource for Oligonucleotide Binding Evaluation) database hosts *S. cerevisiae* data generated by protein binding microarray (PBM) technology. The UniPROBE database provides comprehensive data on the preferences of proteins for any DNA sequence variants of length K (K-mer). For more complete analyses, the UniPROBE website can output weight matrix (PWM) and graphical logo of the K-mer sequence (http://the_brain.bwh.harvard.edu/ uniprobe) (Newburger and Bulyk 2009).
- YEASTRACT DISCOVERER provides tools for *de novo* identification of binding site consensus sequences from a set of gene promoter regions. DISCOVERER contains MUSA (Motif finding using an UnSupervised Approach) algorithms that return the list of motifs ordered by their p-value. In order to extract these motifs, specification of the parameters such as box lengths and distances between boxes is not required from the user (http://www.yeastract.com/formfindregulators.php) (Teixeira *et al*. 2006).
- MYBS (Mining Yeast Binding Sites) integrates both experimentally verified data and predicted position weight matrixes (PWMs) from 11 different databases. To search for motifs in the promoters of the input genes, MYBS uses ChIP-chip data and phylogenetic footprinting as its main filters and considers 7 species including *C. glabrata*. For the identification of potential regulatory associations between two TFs and their combinatorial regulation, MYBS enables the visualization of potential regulators and target gene sets for each TF pair (http://bits.iis.sinica.edu.tw) (Tsai *et al*. 2007).
- YPA (Yeast Promoter Atlas) compiles promoter features of *S. cerevisiae*. YPA integrates various resources (including promoter sequences, TATA boxes, TF binding sites, nucleosome occupancy, DNA bendability, TF-TF interaction and gene expression data) to provide a comprehensive view of gene promoter region (http://ypa.csbb.ntu.edu.tw) (Chang *et al*. 2011).
- JASPAR CORE database contains a curated set of profiles, derived from published collections of experimentally defined TF binding sites for eukaryotes. JASPAR CORE Fungi mostly contains *S. cerevisiae* TF binding site sequence logos (http://jaspar.genereg.net) (Sandelin *et al*. 2004).
- TRANSFAC® provides experimentally-proven eukaryotic TF motif consensus sequences and a list of TF-regulated genes. TRANSCompel contains eukaryotic TF sets that are experimentally proven to interact in a synergistic or antagonistic manner (http://www.gene-regulation.com/pub/databases.html) (Matys *et al*. 2006).

- The MacIsaac collection contains TF motifs from solely ChIP-chip data (MacIsaac *et al*. 2006).

To our knowledge there is no bioinformatics tool available for *C. albicans* promoter region analyses. Hence we generated a *C. albicans* TF binding site database and created Python algorithms to develop publically available software TFbsST (Transcription Factor binding site Search Tool) for the analyses of *Candida* promoter regions. TFbsST is the first bioinformatics tool for *in silico* promoter analyses of *Candida* species including *C. albicans*, *C. dubliniensis*, *C. parapsilosis* and *C. glabrata* (http://bioinfo.ucc.ie/TFbsST/).

To investigate the regulation of *Candida* genes that were altered in the presence of bacterial supernatants, we screened the *C. albicans* gene sets that were upregulated or downregulated in response to *Pseudomonas* supernatants (McAlester *et al*. 2008) across the TFbsST database. After gene ontology (GO) analysis with Blast2Go, the proteins encoded by these genes were grouped according to their localization in the cell. Cell wall proteins are important for biofilm formation. To question their regulation, the promoter regions of the genes coding for the proteins localized around the cell wall were investigated in more detail. The overrepresented TFs were established after analyses of different gene sets and gene families.

4.2 Materials and Methods

4.2.1 General approach

In order to generate a comprehensive TFbsST database for *C. albicans* promoter region analyses *in silico*, we developed a research strategy shown in Figure 1. This workflow represents a modified 'Waterfall' software development model where the project requirements were based on the analyses of DNA sequences in gene promoters. Briefly, to populate a TF database with the corresponding DNA binding site motifs, the relevant literature was reviewed and experimentally validated motifs were selected for the database. Python scripts were designed for promoter analysis and TF database was used as a source of DNA binding site motifs. These files served as the basis for the creation of the TFbsST application. After software design we implemented the testing and verification process that involved prediction of TF frequencies across the *C. albicans* genome and analyses of cell wall protein coding gene families.

To analyse the 4 gene sets, the expression of which was impaired in response to bacterial supernatants according to Holcombe *et al*. (2010), we retrieved their protein sequences from the *Candida* Genome Database (CGD) (Arnaud *et al*. 2005) and annotated with the Blast2Go annotation tool (Conesa *et al*. 2005). Blast2Go uses input protein sequences to assign the proteins into 3 classes according to their molecular function (F), biological process they are involved in (P) and the cellular component (C) where they are localized in the cell. Thus, we defined smaller subsets based on the localisation of the proteins that these differentially expressed genes encoded in the cell. In parallel, as a control group of genes, we downloaded a file with all *C. albicans* genes plus 1000 bp upstream and 1000 bp downstream regions from the CGD and retrieved 1000 bp upstream regions using Python scripts for further TF binding site frequency predictions (%). *In silico* comparative analyses of *C. albicans* and *C. parapsilosis RBT* family gene promoter regions against Efg1p via the TFbsST database validated our computational methodology.

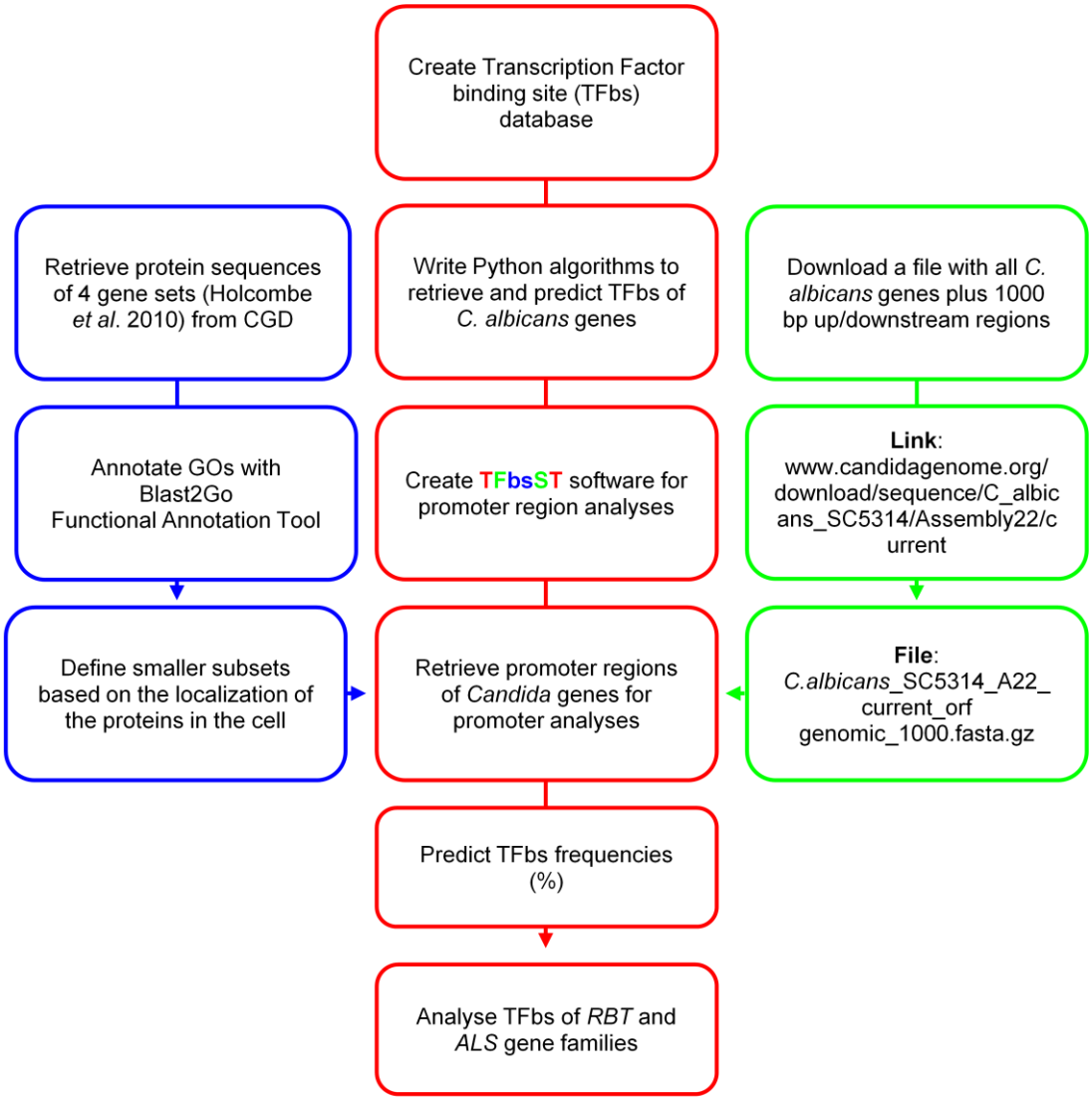

Figure 1| Workflow for the analyses of *C. albicans* gene promoter regions (from -1000 bp to +1 bp). To design TFbsST application we developed TF binding site database and Python algorithms. TFbsST software was used to analyse differentially expressed gene sets of *C. albicans* in response to *P. aeruginosa* supernatants (Holcombe *et al*. 2010). Protein sequences of these gene sets were retrieved from CGD and annotated with Blast2Go annotation tool. Proteins localized across cell wall/surface were considered important due to their significance in biofilm formation. Cell wall/surface group of proteins included *RBT* and *ALS* family genes that were known to be downregulated in presence of bacterial supernatants (Holcombe *et al*. 2010). The groups were further analysed across different TFs of *C. albicans*. A file with all *C. albicans* genes plus 1000 bp upstream and 1000 bp downstream regions served as a control.

4.2.2 Transcription factor binding site database creation

For reliable computational prediction, the ideal TF binding site library would contain TFs associated to a single most accurate DNA binding site motif. Since the minimum number of features increases the prediction power of the results in computational biology, we optimized the TF database relating a single accurate motif to each TF (except from Cwt1p, Msn4p and Srr1p). As a result, our database includes an optimized set of TF binding site motifs that increase the reliability of the predictions. However, the source of each binding site differs for each TF. Hence, it was difficult to compare the TF binding site data. Thus, to select an optimum motif for each TF we took several approaches that lead to the creation of the curated TF binding site library (Table 1).

The TF binding site database was based on publically available experimental data related to *C. albicans* TFs and their binding site motifs (Table 1). To facilitate comparative analysis among *Candida* species we also searched for indicative TF binding sites of *C. parapsilosis*, *C. dubliniensis* and *C. glabrata*. To evaluate the TF binding sites for quality, we applied several approaches. Firstly, a direct search was performed to find DNA binding site motifs of the TFs from the Homann *et al*. (2009) TF mutant collection. Secondly, a general search of the literature was performed to assign binding sites to the corresponding TF, looking for any TF binding sites of *C. albicans* and indicative TF binding sites of *C. parapsilosis*, *C. dubliniensis* and *C. glabrata*. Next, a reference-directed approach was taken to identify the most relevant motifs. For this we prioritised the motifs that were found by the reliable methods, for paradigm ChIP-Chip analysis, and were published in established journals. To optimise our database, we also took a time-directed approach considering the most recently identified binding sites. Some TF binding sites of *C. albicans* and *S. cerevisiae* display similarity in their sequence. Finally, we compared *C. albicans* TF binding sites with that of *S. cerevisiae* for similarities in YeTFaSCo (de Boer and Hughes 2011). YeTFaSCo contains a *S. cerevisiae* TF collection of specificities that are evaluated for quality with several metrics.

Table 1| ***Candida*** **transcription factor binding site consensus sequences**. ID shows the systematic nomenclature as in CGD and TF indicates the name of a transcription factor.

ID	TF	TF consensus binding site	%[a]	Genes[b]	Reference
Candida albicans					
CR_07440W	Ace2p	HHCACCACCWM	12	746	Swidergall *et al*. (2015)
C3_06000W	Ahr1p	CGNBWVWWVNT	60	3731	Askew *et al*. (2011)
C1_09090C	Arg81p	KCGCGST	3	187	Tuch *et al*. (2008)
CR_02560C	Asg1p (asgs)	YMTTGKYS	78	4851	Tuch *et al*. (2008)
CR_02510W	Azf1p (orf19.173)	RAADAARAAR	91	5659	Swidergall *et al*. (2015)
C2_05640W	Bas1p	TGACTC	27	1679	Gasch *et al*. (2004)
CR_06440C	Bcr1p	TAMATRCATR	5	311	Nobile *et al*. (2012)
C2_01110C	Bcy1p (Mcb)	ACGCG	18	1119	Gasch *et al*. (2004)
C1_05140W	Brg1p (Gat2)	SMGGTAM	29	1804	Nobile *et al*. (2012)
C3_02220W	Cap1p (Ap-1)	MTKASTMA	41	2550	Goudot *et al*. (2011)
C4_06580W	Cbf1p	TCACGTG	9	560	Gasch *et al*. (2004)
C1_07370C	Cph1p (ScSte12)	TGAAACA	30	1866	Banerjee *et al*. (2007)
C6_00280W	Cph2p (Sre1-like seq)	YCACMCCAY	6	373	Lane *et al*.(2001); Lane *et al*. (2015)
CR_05530C	Cwt1p	AGGGCT	10	622	Moreno *et al*. (2007)
		AGCCCT			Moreno *et al*. (2007)
CR_07530C	Ecm22p/Upc2p	TCGTWWWW	49	3047	Gasch *et al*. (2004)
CR_07890W	Efg1p	RTGCATRW	17	1057	Nobile *et al*. (2012)
C3_00670C	Fkh2p	RTAAAYAWW	61	3794	Gasch *et al*.(2004); Gordan *et al*. (2012)
C2_09940W	Gcn4p	TGACTM	55	3420	Gasch *et al*. (2004)
C1_07680W	Hap2p	CCAATCA	22	1368	Ozsarac *et al*. (1995)
C4_01390W	Hap3p	CCAAT	96	5970	Johnson *et al*. (2005); Baek *et al*. (2008); Linde *et al*. (2010)
C1_09870W	Hcm1p (Fkh2)	WMAAYA	100	6219	Gordan *et al*. (2012)
C2_03840C	Ino4p (Ino2+Ino4)	NBWTCASRTG	15	933	Hoppen *et al*. (2007)
C3_04110C	Mbp1p	ACGCGTSR	2	124	Gordan *et al*. (2012)
C7_00890C	Mcm1p	ACCRRAWWRGGMA	1	62	Perez *et al*. (2014)
C2_10230W	Met32p	TGTGGC	23	1430	Gasch *et al*. (2004)
C5_02940C	Mig1p	SYGGRG	65	4042	Banerjee *et al*. (2007)
C1_08940C	Msn4p (STRE - Msn2/4)	CCCCT	55	3420	Banerjee *et al*. (2007)
		AGGGG			Banerjee *et al*. (2007)
C5_01755C	Matα1p(MtlALPHA1)	WTCCTTW	62	3856	Baker *et al*. (2011); Tuch *et al*. (2008)
C2_00140W	Ndt80p	TTACACAAA	5	311	Nobile *et al*. (2012)
C7_04230W	Nrg1p	MVCCCT	68	4229	Argimon *et al*. (2007); Banerjee *et al*. (2007)
CR_02640W	Rfg1p	ACAAT	99	6157	Gordan *et al*. (2012)
C1_14340C	Rim101p	CCAAGAA	28	1741	Ramon and Fonzi (2003)
C1_13620W	Rob1p	GGWAAWNWAWWTCC	1	62	Nobile *et al*. (2012)
C1_04330W	Rpn4p	GGTGGCAAAA	1	62	Gasch *et al*. (2004)
C1_10020W	Sfu1p	WGATAA	91	5659	Linde *et al*. (2010)
CR_05610C	Srr1p (SRR)	AAGAA	100	6219	Banerjee *et al*. (2007)
		CCGAA			Banerjee *et al*. (2007)
		ATTGG			Banerjee *et al*. (2007)
C1_01790W	Swi4p	CRCGAA	27	1679	Gordan *et al*. (2012)
C5_01840C	Tac1p	CGGAWATCGGATATTTTTTTT	0.1	6	Banerjee *et al*. (2007)
C3_04530C	Tec1p	RCATTCY	35	2177	Nobile *et al*. (2012); Argimon *et al*. (2007)
C1_08460C	Upc2p	CGBDTR	91	5659	Znaidi *et al*. (2008)
C1_10150W	Wor1p	WTARRSTTT	30	1866	Lohse *et al*. (2010)
C7_00970C	Yox1p	WWYAWTT	100	6219	Tuch *et al*. (2008)
Candida parapsilosis **CPAR2**					
_	Dal82p	AYGCRC			Connolly *et al*. (2013)
213640	Ndt80p	CACAAAR			Connolly *et al*. (2013)
701620	Efg1p	CTGCATR			Connolly *et al*. (2013)
100880	Azf1p	AAAARDA			Connolly *et al*. (2013)
403080	Zap1p	CACBACC			Connolly *et al*. (2013)
211740	Stp4p	GGTAGCR			Connolly *et al*. (2013)
Candida dubliniensis					
Cd36_07150	Hap2P	CCAAT			CGD
Candida glabrata					
CAGL0A00451g	Pdr1p	HYCCRKGGR			Paul *et al*. (2014)

[a] Frequency of the TFs among all *C. albicans* (SC3514) genes (~6219) is given in % that is rounded to its closest digit (This study). The analyses were carried out from December 2015 to January 2016.

[b] Number of *C. albican*s genes predicted to possess a TF binding site (This study).

4.2.3 Python algorithm design

To analyze the promoter regions (1000 bp upstream) of *Candida* genes, Python algorithms were designed using mainly 'Bio' and 'Bio.Seq' libraries as well as 'Seq', 'SeqIO', 'sys' and 're' modules of Python 3.2 and 2.7 versions (www.python.org). Two similar scripts were designed to answer two different biological questions. The first script predicted the frequency (%) of the input genes possessing a selected TF. However, for more detailed analysis the second script was created using the first as a template. The second script identified a DNA binding site motif, sequence ID where this binding site was present, the exact start position of the binding site, as well as a total number of the binding sites in each sequence.

For both algorithms an input file had a FASTA format with '>' sign followed by a gene ID, and a DNA sequence starting on the next line. The input file was treated as a dictionary with the IDs and their sequences defined as the keys and values respectively. Another dictionary was required for the TF database where a TF was defined as the key and its corresponding binding sites as a string of values. To decode the motifs written with ambiguous DNA code an incorporated combinatorial program was designed and called from the main script.

To output the percentage of sequences possessing a specific TF binding site, we followed simple mathematical logic. To obtain TF information, its copy was made and the value of each TF (key) was zeroed. Next, to define a sequence line and the reverse complementary DNA strand, the sequences and TFs were looped in the file creating a sequence counter. To check whether a specific binding site matched with any sequence, a 'match' statement was used in combination with 'for' and 'in' statements. When the motif was found in the sequence the count was increased by 1. To output the percentage count, the final statement was iterated through the TF count data.

To retrieve the detailed information about the binding site motif, the sequence ID where this motif was present, the exact start position of the motif and a total number of binding sites in each sequence, a similar logic was followed with minor changes. The above script that outputted the percentage data was modified and the output was differentiated towards the final 'print' statement. Namely, when more than 0 binding sites were found in the sequence, the program outputted the sequence, TF, binding site motif as well as the start position and a total number of the binding sites for each DNA strand.

4.2.4 TFbsST website development

To avail comprehensive *in silico* analyses of *Candida* promoter regions we went beyond the Python scripts and created the TFbsST database (Transcription Factor binding site Search Tool). TFbsST was based on the TF binding site database (Table 1) and Python scripts (see above) for promoter region analyses following a 'Waterfall' model of software development. The Waterfall model includes 5 elements: requirements, design, implementation, verification and maintenance. To develop the TFbsST software we mainly used HTML5 (Hyper Text Markup Language 5), mySQL (Structured Query Language) and JavaScript programming languages that incorporated different text files. These text files stored data for TF binding site motifs, *Candida* species and references related to each motif. This flexible design simplified the upgrading and maintenance processes of the TFbsST website. TFbsST logos were designed using the free online software (www.logomakr.com).

4.2.5 GO annotation

To analyze *Candida* genes that were altered in expression in response to bacterial supernatants, we annotated their Gene Ontologies (GOs). More specifically, the protein sequences of *C. albicans* genes upregulated or downregulated in presence of *P. aeruginosa* supernatants, were retrieved from CGD and annotated with the Blast2Go functional annotation tool (Conesa *et al*. 2005). Blast2Go uses input protein sequences to assign the proteins into 3 classes according to their molecular function (F), biological process they are involved in (P) and the cellular component (C) where they are localized in the cell. Cellular component (C) annotation was used to analyze the localization of proteins whose gene expression was impaired after application of bacterial supernatants. The corresponding graphs were performed using Prism V6 for Mac (www.graphpad.com) or R Statistical Software (R development core team).

4.2.6 *Candida albicans* and *Candida parapsilosis* promoter analysis

Cell wall proteins are important for biofilm formation. In order to validate our computational method, we analysed the promoter regions of the *RBT* genes coding for the cell wall proteins in two *Candida* species, *C. albicans* and *C. parapsilosis*. All *RBT* family genes with their promoter regions were retrieved and stored in a single FASTA file for each *Candida* species. To extract and store the promoter regions from each file, we used Python scripts. *RBT* family gene promoter regions were analysed against Efg1p binding sites of the corresponding *Candida* species via the TFbsST database. The screening option of the TFbsST

database that availed analytical analysis of the specified promoters produced the graphical output of the TF binding site positions in addition to other details. These details included the ID of the promoter sequence, TF, TF binding site motif and its start position as well as the total number of binding sites in each promoter. To design the TF binding site logos corresponding to each set of *RBT* family gene promoter, we used a Weblogo designer tool (http://weblogo.berkeley.edu/logo.cgi) incorporated into the TFbsST database.

4.3 Results

4.3.1 Transcription factor binding site database

To analyse *C. albicans* promoter regions a detailed plan was designed as shown in the Figure 1. Initially, to develop a TFbsST application, a TF binding site database was generated from the published experimentally defined motifs. Python algorithms were designed following a modified 'Waterfall' software development strategy. In order to analyse *C. albicans* 4 gene sets of Holcombe *et al*. (2010), with upregulated or downregulated gene expression in response to bacterial supernatants, we retrieved their protein sequences from CGD and annotated their GOs using the Blast2Go annotation. Thus, we defined smaller protein subsets based on the localisation of the proteins that these differentially expressed genes encoded in the cell. As a control group of genes, we downloaded a file with all *C. albicans* genes with 1000 bp up/downstream regions (CGD) and retrieved their promoters using Python scripts. To evaluate the TFbsST database we predicted the TF frequencies (%) in *C. albicans* genome and carried out promoter analyses of the cell wall protein coding family genes, *RBT* and *ALS*. Comparative analyses of *C. albicans* and *C. parapsilosis RBT* family gene promoters against Efg1p validated our approach.

TF binding site database was based on the published literature (Table 1). In total, we found binding site motifs for 41 TFs and 1 TF complex (Ino2p+Ino4p) in *C. albicans*. To facilitate comparative analysis among *Candida* species we also searched for indicative TF binding sites of *C. parapsilosis*, *C. dubliniensis* and *C. glabrata*. TFbsST contains 4 tables storing TF binding site data that are tagged with the corresponding *Candida* species (Table 1). For more precise presentation of the TFs, their updated systematic nomenclature was included in the first column of the Table 1. The second column displays a name of each TF and the third consensus binding site motif written with IUPAC nucleotide code (for abbreviations see www.genome.jp/kegg/catalog/codes1). To better understand the distribution of the TFs in *C. albicans* genome, we calculated TF frequencies (fourth column) and the approximate number of genes (fifth column) regulated by each TF. The final column refers to the relevant publication from where the TF binding site motif was retrieved (Table 1).

The preliminarily generated TF binding site database was further optimised to create a TF binding site library with a single binding site corresponding to each TF. However, in some cases multiple motifs were associated with one TF, for example, Cwt1p, Msn4p and Srr1p. Several motifs possessed flanking or internal bases with low information content. Since a

motif is not improved by removing these bases (de Boer and Hughes 2011), most binding site motifs were left in their original form in our database.

4.3.2 TFbsST software

TFbsST was based on the TF binding site database (Table 1) and the Python algorithms (see Materials and methods) that were designed for promoter analyses. The Python algorithms use the TF database as a source of motifs for DNA sequence analysis. The home page of the TFbsST website displays the basic information about the software and its function (Figure 2A). A left hand-side (LHS) menu bar was created to facilitate rapid navigation within the website. The LHS bar contains links to access DNA sequence analysis tool (Scan) (Figure 2B), downloadable table of the TF motif database (Motifs), downloadable list of publications where each motif was found (References) (also see Table 1) and an option allowing addition of a new TF binding site (Add Motif). The TFbsST database can also be used to access 7 similar databases listed in the 'Databases' section of the LHS bar (Figure 2C). The last features of the LHS menu include the information about the authors (Authors) and contacts (Contact us) (Figure 2).

The main function of the TFbsST application is to analyse the DNA sequences across all the TFs available in its database. A DNA sequence analysis tool located under the 'Scan' link leads to the next page where a desired *Candida* species can be selected. The TFbsST website can be used to analyze promoter regions of *C. albicans* and related species including *C. parapsilosis, C. dubliniensis* and *C. glabrata*. Each *Candida* species is linked to a different database table availing more information about *C. albicans* (Table 1). The sequence scanner of the TFbsST website outputs 2 different results based on the users' request. The first option outputs a table with the percentage (%) of the user-defined sequences possessing a selected TF or all the available TFs of the database. To calculate the frequency (%), a number of genes with predicted TF is divided by the total number of input genes and multiplied by 100. The second option outputs a graphical display of the precise binding site position(s) of a selected TF among other details. These details are tabulated and include the ID and strand of input promoter sequence where any binding site is found, binding site motif and the corresponding TF.

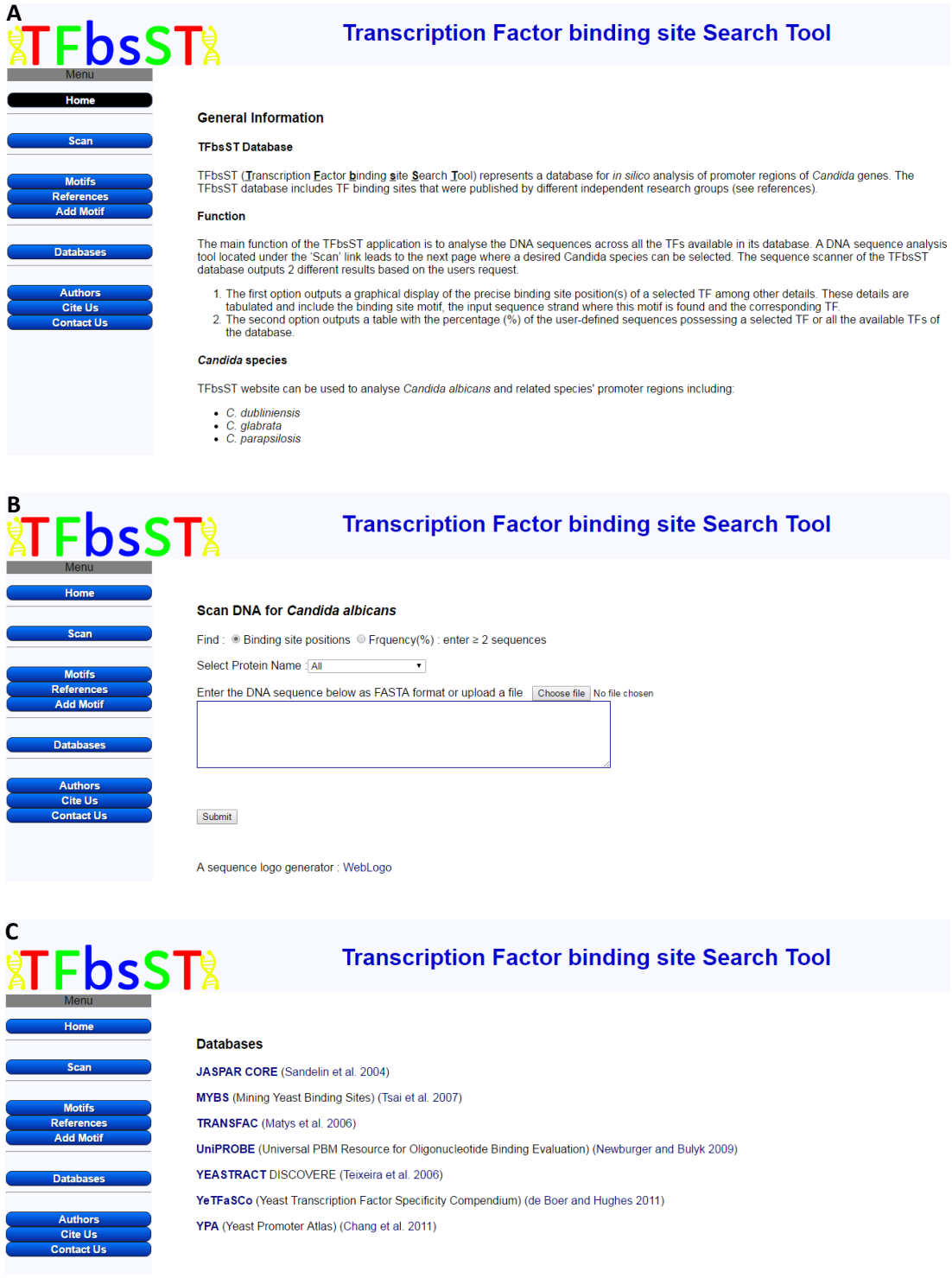

Figure 2| Screenshots illustrating some features of the TFbsST database. **A**| Part of the TFbsST database Homepage. The Homepage provides general information about the database and its functionalities. LHS menu facilitates rapid navigation within the website through the Scan, Motifs, References, Databases and other features. **B**| Scan function of the TFbsST database. This function facilitates DNA sequence analysis for the TFs of the TFbsST database with the 'Binding site position' and 'Frequency' options. The results of the

'Binding site position' analyses are summarised in graphs and tables, the 'Frequency' analyses are organised in tables. **C**| Databases listed in the TFbsST database. The TFbsST database provides an access to the 7 TF databases that mainly focus on *S. cerevisiae*.

4.3.3 Overrepresented and underrepresented transcription factors in *Candida albicans* gene promoter regions

To analyze *C. albicans* promoter regions we used TFbsST application and Python programs. The Python scripts that analysed the TF frequencies and predicted the TF binding sites were incorporated into the TFbsST software. However, some Python algorithms were additionally designed to retrieve gene promoter regions (-1000 bp to +1 bp). *In silico* genome-wide frequency analyses of *C. albicans* promoters against TFbsST database predicted that 3 TFs Hcm1p, Srr1p and Yox1p possessed ubiquitously distributed binding sites across all its 6219 genes (100%) (Table 1). However, these TFs are unlikely to regulate the whole *C. albicans* genome *in vivo*, hence more work is required to optimise their binding site motifs. The DNA binding sites of 5 TFs, Tac1p, Rob1p, Rpn4p, Mcm1p and Mbp1p, were predicted to appear sporadically (1% - 2%) in *C. albicans* promoters occupying about 100 genes. Such a low occurrence frequency indicates that the above TFs regulate specific group of genes (Table 1).

4.3.4 *RBT* and *ALS* are important for interaction between *Candida albicans* and *Pseudomonas aeruginosa*

To better understand the regulation of genes whose expression was impaired in response to bacterial supernatants we carried out *in silico* promoter analyses via the TFbsST database. Previous studies of Holcombe *et al*. (2010) and McAlester *et al*. (2008) had established differentially expressed genes of *C. albicans* in response to *P. aeruginosa* supernatants. In Holcombe *et al*. (2010) study, *C. albicans* cultures were grown in presence of 4 different types of *Pseudomonas* supernatants (wild-type PAO1, clinical isolates CF144, CF177 and HSL-free mutant PAO1ΔQS) as well as in presence of supernatants derived from HSL-producing *Pseudomonas* strains. Gene expression profiles of *Candida* indicated that some genes were significantly upregulated or downregulated in the above conditions (threshold $\geq$2 fold) (Holcombe *et al*. 2010). Promoter regions of these gene sets were subject to TF frequency analysis (Table 2). The results of computational investigation revealed that Efg1p and Mbp1p were overrepresented in the downregulated sets and Ndt80p in the upregulated sets of the genes treated with all 4 types of bacterial

supernatants (Table 2). All three TFs regulate biofilm development in *C. albicans* (Nobile *et al*. 2012). This prediction suggested that the pathways of *C. albicans* - *P. aerugionsa* communication and *Candida* biofilm formation may converge. This seems logical since we examine biofilm formation as a reference point of the above two species interaction. A file with all *C. albicans* gene promoter regions served as a control.

To further investigate the interaction between *C. albicans* and *P. aeruginosa*, we annotated *C. albicans* gene sets that were significantly upregulated or downregulated in response to bacterial supernatants. We predicted subcellular localization of the proteins that these differentially expressed genes encoded in the cell. Protein localization is an important component of computational prediction informing where a protein resides in the cell and what is its function based on its sequence. The protein sequences of differentially expressed genes were retrieved from CGD and their GOs were annotated with Blast2Go annotation tool. With this up-to-date annotation we were able to annotate many proteins that were previously assigned to unknown function. For instance, 13 proteins with previously unknown function, encoded by the upregulated genes in presence of 4 types of bacterial supernatants, were annotated in this study (Appendix 4.5 Table 6 bold type). Many genes that were downregulated in presence of bacterial supernatants were also encoding for proteins with previously unknown function (Holcombe *et al*. 2010; McAlester *et al*. 2008). From this list 20 additional genes were annotated in this study (Appendix 4.5 Table 7 bold type). Recent annotations of the following genes *KAR4*, orf19.1336.2, orf19.6747, orf19.1114, orf19.409 (Appendix 4.5 Table 8 bold type) and *POL12, FRK1, BUD14, WOR3, NOP9* (Appendix 4.5 Table 9 bold type) provide further insights about the proteins that are involved in *Candida – Pseudomonas* interactions.

Blast2Go is a sequence-based annotation tool that assigns the proteins into 3 categories according to their molecular function (F), biological process they are involved in (P) and the cellular component where they are localized in the cell (C). Using cellular component-based localization annotation, we shortlisted smaller gene subsets coding for cell wall/surface proteins since they are important for *Candida* biofilm formation (Figure 3 and Table 3). Notably, downregulated genes coding for proteins of cell wall/surface, plasma membrane, endoplasmic reticulum and intracellular region exceeded those of upregulated genes in the gene set treated with 4 different types of bacterial supernatants (Figure 3A). These cell wall/surface protein coding genes contained two members of *RBT* and *ALS* family genes, *RBT1*, *RBT4* and *ALS1, ALS3* respectively (Table 3). Clearly, GO annotation confirmed that

RBT and *ALS* gene families are important for interaction between *C. albicans* and *P. aerugionsa*.

Table 2| Promoter region (from -1000 bp to +1 bp) analyses of *C. albicans* genes that were impaired after application of *P. aeruginosa* QS molecules.

TF	Control [a] (%)	All 4 types of bacterial supernatants				HSL-containing supernatants			
		Up [b] (%)	Up S [c] (%)	Down [b] (%)	Down S [c] (%)	Up [b] (%)	Up S [c] (%)	Down [b] (%)	Down S [c] (%)
Ace2	12	10	20	10	8	6	0	8	0
Ahr1	60	61	40	62	86	71	100	58	0
Arg81	3	3	10	6	0	3	0	0	0
Asg1 (asgs)	78	75	80	84	100	74	80	93	100
Azf1	91	94	90	92	93	90	100	93	50
Bas1	27	27	30	40	36	43	20	12	0
Bcr1	5	8	10	3	0	8	0	4	0
Bcy1 (Mcb)	18	11	20	21	22	16	20	12	0
Brg1 (Gat2)	29	29	40	20	29	27	40	16	50
Cap1 (Ap-1)	41	42	50	57	58	48	80	27	0
Cbf1	9	16	10	7	8	24	20	4	50
Cph1 (ScSte12)	30	29	10	26	15	32	40	16	50
Cph2 (Sre1-like seq)	6	5	10	5	8	8	20	8	0
Cwt1	10	19	20	13	15	6	0	8	50
Ecm22/Upc 2	49	47	40	55	86	35	40	70	50
Efg1	**17**	**15**	**20**	**20**	**43**	**16**	**20**	**12**	**0**
Fkh2	61	69	80	70	79	48	40	62	50
Gcn4	55	60	70	60	65	77	60	35	100
Hap2	22	17	0	23	29	19	40	20	50
Hap3	96	49	100	92	100	95	80	81	50
Hcm1 (Fkh2)	100	100	100	100	100	100	100	100	100
Ino4 (Ino2+Ino4)	15	18	10	15	22	27	20	12	0
Mbp1	**2**	**0**	**0**	**5**	**15**	**0**	**0**	**4**	**0**
Mcm1	**1**	**0**	**0**	**0**	**0**	**0**	**0**	**0**	**0**
Met32	23	25	20	25	22	24	0	20	0
Mig1	65	60	30	65	58	69	40	54	0
Msn4 (STRE - Msn2/4)	55	51	60	65	50	45	0	58	50
Matα1 (MTLALPHA 1)	62	60	50	71	79	66	80	62	100
Ndt80	5	7	10	10	8	8	40	12	0
Nrg1	68	70	80	70	79	66	80	70	100
Rfg1	99	99	100	100	100	100	100	97	100
Rim101	28	27	40	29	36	19	0	47	50
Rob1	**1**	**1**	**0**	**0**	**0**	**0**	**0**	**0**	**0**
Rpn4	**1**	**1**	**10**	**0**	**0**	**3**	**0**	**0**	**0**
Sfu1	91	92	90	93	86	85	80	89	50
Srr1 (SRR)	100	100	100	100	100	100	100	100	100
Swi4	27	17	10	24	36	16	0	20	0
Tac1	**0.1**	**0**	**0**	**0**	**0**	**0**	**0**	**0**	**0**
Tec1	35	34	70	38	65	37	60	47	0
Upc2	91	94	100	92	100	93	80	89	100
Wor1	30	30	20	27	22	27	40	39	50
Yox1	100	100	100	100	100	100	100	100	100

[a] Control includes all *Candida albicans* (SC3514) genes (~6219 genes).

[b] Gene sets were retrieved from Holcombe *et al*. (2010) (Supplementary Tables S1 – S4).

[c] Subsets are Cell wall/surface genes/proteins from the corresponding gene set (see Figure 2).

The frequencies (%) are rounded to their closest digit (This study). The analyses were carried out from December 2015 to January 2016.

Underrepresented TFs in the control group are given in **bold** type (This study).

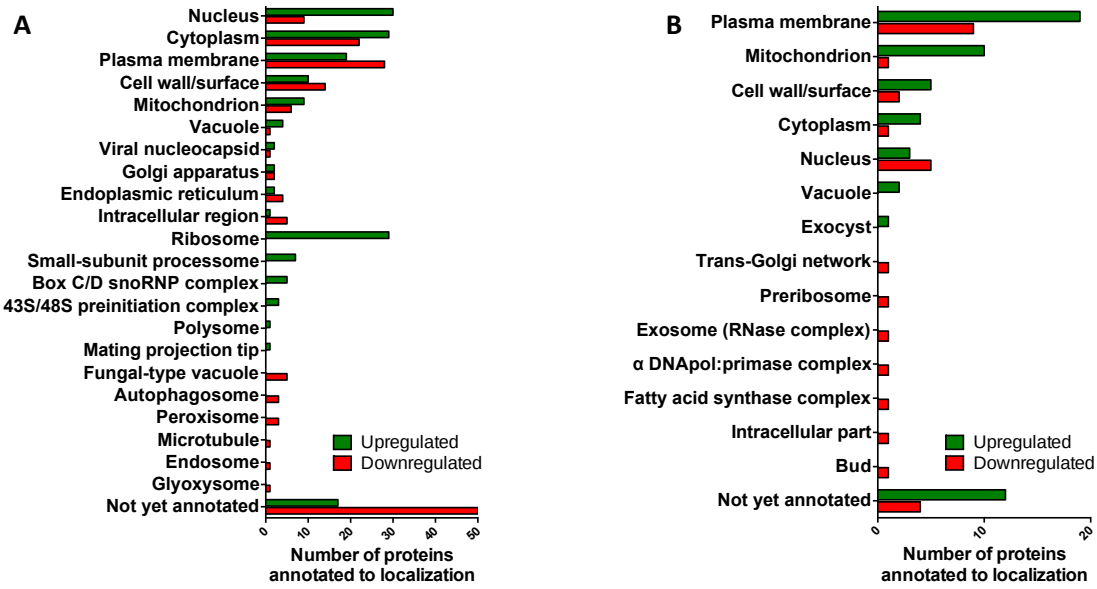

Figure 3| Localization of *C. albicans* proteins encoded by the upregulated (green bars) and downregulated (red bars) genes in response to *P. aeruginosa* supernatants. **A**| Genes with altered expression in response to supernatants derived from 4 strains of *P. aeruginosa* (wild-type PAO1, clinical isolates CF144, CF177 and HSL-free PAO1ΔQS). **B**| Genes with altered expression in response to supernatants derived from HSL-producing *P. aeruginosa* strains. Image was produced using scientific statistics software GraphPad Prism v 6 for Mac.

Table 3| *C. albicans* gene subsets coding for cell wall/surface proteins. These subsets were either upregulated or downregulated in response to the supernatants derived from 4 strains of *Pseudomonas*, PAO1, CF144, CF177, PAO1ΔQS and HSL-containing supernatants (data were extracted from Figure 3).

4 types of bacterial supernatants				**HSL-containing supernatants**			
Upregulated		**Downregulated**		**Upregulated**		**Downregulated**	
ID	**Gene**	**ID**	**Gene**	**ID**	**Gene**	**ID**	**Gene**
CR_10110W	*CHT3*	C4_02410C	*AHP1*	C6_03700W	*ALS1*	C4_03570W	*HWP1*
C2_08490W	*DSE1*	CR_07070C	*ALS3*	C4_01160W	*CRD2*	C2_01380W	*PLB4.5*
C5_02080C	*HSP12*	C4_03470C	*ECE1*	C5_02790C	*GAP1*		
C4_06720W	*NOP1*	C5_02460C	*ECM331*	C6_02010C	*GPD2*		
C2_08870C	*PIR1*	C6_00440C	*FET34*	C4_04080C	*PGA31*		
C6_02070C	*RPL23A*	C1_14130W	*FTR1*				
C2_08040C	*RPS10*	CR_10100C	*INO1*				
C6_00650C	*RPS13*	C3_01360C	*IRO1*				
C5_04110W	*SCW11*	CR_10790W	*MAL2*				
C2_08590W	*YWP1*	C4_00200C	*MET15*				
		C4_03520C	*RBT1*				
		C1_07030C	*RBT4*				
		C2_00680C	*SOD5*				
		C7_00260C	*YLR001C*				

4.3.5 Overrepresented TFs in the *RBT* and *ALS* family gene promoters

Previous studies of Holcombe *et al*. (2010) had shown that *RBT1* and *RBT4* were downregulated in presence of bacterial supernatants. From Holcombe *et al*. (2010) dataset *RBT1* and *RBT4*, along with *ALS1* and *ALS3* (although not downregulated), were also found among our shortlisted genes coding for cell wall proteins (Table 3). Hence, it was interesting to explore which TFs could possibly regulate expression of these genes. To predict TF distribution frequencies across *C. albicans RBT* and *ALS* family gene promoters we retrieved their gene sequences plus 1000 bp up/downstream regions from CGD. The promoters of *RBT* family genes, including *RBT1, RBT2, RBT4, RBT5, RBT6, RBT7* and *RBT8*, were retrieved with Python scripts and analysed for TF frequencies in the TFbsST software. The promoter regions of *ALS* family genes, *ALS1, ALS2, ALS3* (*CDC24*)*, ALS3, ALS4, ALS5, ALS6, ALS7* and *ALS9*, were analysed in the same way using a separate FASTA file. Figure 4 shows predicted TF ratios in the promoters of the *RBT* and *ALS* family genes (Figure 4). *RBT* family possessed 7 overrepresented TFs, Mcm1p, Mbp1p, Bcr1p, Efg1p, Ndt80p, Cwt1p and Cph2p, determined by the 2-fold threshold (red horizontal line). Only 2 TFs, Rpn4p and Efg1p, were overrepresented in the *ALS* family. Notably, Efg1p was overrepresented in both gene families (Figure 4).

More detailed analyses showed that 3 out of 9 master regulators, Efg1p, Bcr1p and Ndt80p, that govern biofilm development in *C. albicans* (Nobile *et al*. 2012; Fox *et al*. 2015), possessed binding sites across promoter regions of 5 different genes in the *RBT* family (Table 4). Table 4 shows that *RBT1*, *RBT4* and *RBT6* are mainly regulated by Efg1p, *RBT2* is additionally regulated by Bcr1p and *RBT5* solely by Bcr1p and Ndt80p. DNA binding sites of Efg1p were also found in 4 *ALS* family genes, *ALS1, ALS3, ALS4* and *ALS9* (Table 5). Thus, Efg1p appeared as a protagonist of gene regulation in both gene families, *RBT* and *ALS*. Other overrepresented TFs in the *RBT* family were Cph2p and Cwt1p. Cph2p promotes hyphal growth by directly regulating Tec1p to induce hyphal-specific genes (Lane *et al.* 2001) and Cwt1p regulates cell wall integrity (Moreno *et al*. 2003). These results partially confirmed our approach since *RBT* family genes are strongly expressed during hyphal growth but they are dramatically downregulated in presence of *Pseudomonas* supernatants under yeast growth conditions (McAlester *et al*. 2008; Holcombe *et al*. 2010).

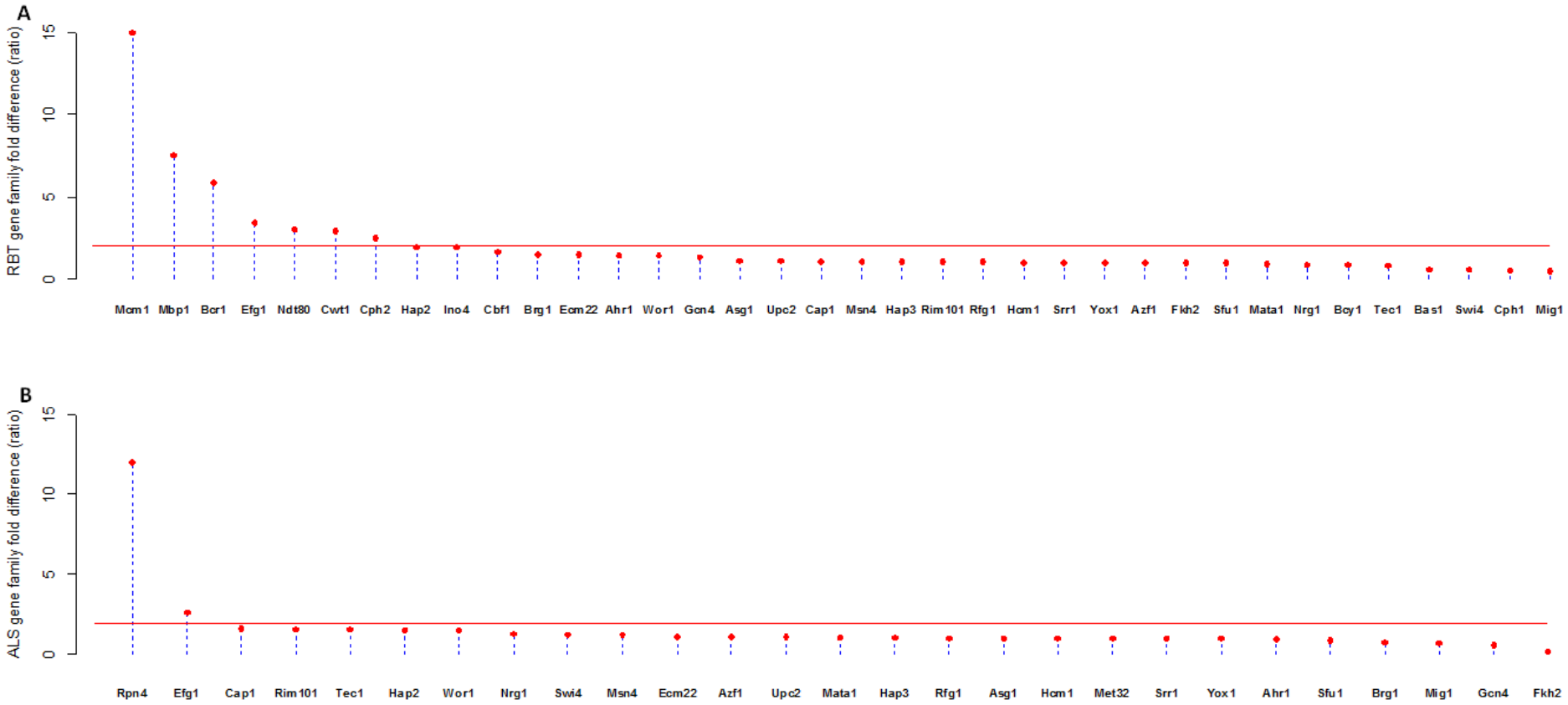

Figure 4| **Promoter region (from -1000 bp to +1 bp) analyses of the *RBT* and *ALS* gene families across the TFbsST database**. Individual TF frequency (%) of each gene family member was divided by the TF frequency of the control group (all *Candida* genes) to obtain the final ratio illustrated with red circles. **A**| *RBT* and **B**| *ALS* gene families. A set of all *C. albicans* genes was used as a control. Overrepresented TFs are determined by the empirical 2-fold threshold (red horizontal line). Efg1p was overrepresented in both gene families. The figures were produced using the R Statistical Software.

Table 4| Promoter region (from -1000 bp to +1 bp) analyses of *C. albicans* genes.

TF binding site	*RBT1*	*RBT2*	*RBT4*	*RBT5*	*RBT6*	*RBT7*	*RBT8*
Ahr1	+		+	+	+	+	+
Asg1 (asgs)	+	+	+	+		+	+
Azf1	+	+	+	+	+	+	
Bas1					+		
Bcr1		**+**		**+**			
Bcy1 (Mcb)			+				
Brg1 (Gat2)	+			+		+	
Cap1 (Ap-1)	+	+		+			
Cbf1							+
Cph1 (ScSte12)			+				
Cph2 (Sre1-like seq)						**+**	
Cwt1		**+**				**+**	
Ecm22/Upc2	+			+	+	+	+
Efg1	**+**	**+**	**+**		**+**		
Fkh2	+		+			+	+
Gcn4	+	+	+	+	+		
Hap2		+				+	+
Hap3	+	+	+	+	+	+	+
Hcm1 (Fkh2)	+	+	+	+	+	+	+
Ino4 (Ino2+Ino4)				+			+
Mbp1			**+**				
Mcm1							**+**
Mig1	+		+				
Msn4 (STRE - Msn2/4)		+	+	+		+	
Matα1 (MTLALPHA1)		+	+	+	+		
Ndt80				**+**			
Nrg1		+	+		+	+	
Rfg1	+	+	+	+	+	+	+
Rim101				+	+		
Sfu1	+	+	+		+	+	+
Srr1 (SRR)	+	+	+	+	+	+	+
Swi4							+
Tec1	+		+				
Upc2	+	+	+	+	+	+	+
Wor1			+		+	+	
Yox1	+	+	+	+	+	+	+
Total TF binding sites	18	18	22	19	17	19	16

Overrepresented TFs in relation to the whole *C. albicans* genome determined by the 2-fold threshold, are given in **bold** type (This study). Data are extracted from Figure 4.
RBT gene family includes: *RBT1, RBT2 (FRE10), RBT4, RBT5, RBT6 (PGA7), RBT7* and *RBT8 (PGA10).*

Table 5| Promoter region (from -1000 bp to +1 bp) analyses of *C. albicans* genes.

TF binding site	*ALS1*	*ALS2*	*ALS3*	*ALS3*[a]	*ALS4*	*ALS5*	*ALS6*	*ALS7*	*ALS9*
Ahr1	+	+			+	+		+	
Asg1 (asgs)	+	+	+	+			+	+	+
Azf1	+	+	+	+	+	+	+	+	+
Brg1 (Gat2)						+			+
Cap1 (Ap-1)	+	+	+	+	+				+
Ecm22/Upc2	+	+	+			+		+	
Efg1	**+**		**+**		**+**				**+**
Fkh2		+							
Gcn4		+		+		+			
Hap2				+		+			+
Hap3	+	+	+	+	+	+	+	+	+
Hcm1 (Fkh2)	+	+	+	+	+	+	+	+	+
Met32								+	+
Mig1					+	+	+		+
Msn4 (STRE - Msn2/4)		+	+	+	+	+	+		
Matα1 (MTLALPHA1)	+	+	+		+		+		+
Nrg1	+	+	+	+	+	+	+		+
Rfg1	+	+	+	+	+	+	+	+	+
Rim101		+		+	+				+
Rpn4				**+**					
Sfu1	+	+	+		+	+	+		+
Srr1 (SRR)	+	+	+	+	+	+	+	+	+
Swi4		+		+	+				
Tec1	+	+	+				+		+
Upc2	+	+	+	+	+	+	+	+	+
Wor1	+			+		+	+		
Yox1	+	+	+	+	+	+	+	+	+
Total TF binding site	17	20	16	17	17	17	15	11	19

[a] *ALS3* (*CDC24*).

Overrepresented TFs in relation to the whole *C. albicans* genome, determined by the 2-fold threshold, are given in **bold** type (This study). Data are extracted from Figure 4.

ALS gene family include: *ALS1, ALS2, ALS3* (*CDC24*), *ALS3, ALS4, ALS5, ALS6, ALS7* and *ALS9.*

4.3.6 Efg1p binding sites in *Candida albicans* and *Candida parapsilosis* promoter regions

In order to validate our database we studied abundance of Efg1p binding sites in a related yeast *C. parapsilosis*. Efg1p is a well-studied TF regulating hyphal growth, adhesion and virulence. Efg1p was overrepresented in the promoter regions of the *RBT* and *ALS* gene families in *C. albicans* (Figure 4). Hence, we questioned whether Efg1p could regulate *RBT* family genes across other *Candida* species. To answer this question we studied the abundance of Efg1p binding sites in the promoters of the *C. parapsilosis RBT* family genes (Figure 5). Both *C. albicans* and *C. parapsilosis* possess 7 *RBT* family genes. Gene sequences plus 1000 bp up/downstream regions of all *RBT* family genes were retrieved from CGD and stored in FASTA files. Upstream (1000 bp) promoter sequences were extracted via Python programs and their detailed analyses were conducted using a Binding site position search option of the TFbsST application. TFbsST outputted detailed results of the search (Figure 5A). Figure 5A shows that Efg1p binding sites were found in both *C. albicans* and *C. parapsilosis* (Figure 5A). More specifically, *C. albicans* possessed Efg1p binding sites in 4 *RBT* gene promoters, *RBT1, RBT2* (including reverse-complement strand), *RBT4* and *RBT6*. *C. parapsilosis* contained Efg1p binding site motifs in 2 *RBT* gene promoters, *RBT2* and *RBT4*. To compare Efg1p motifs we produced representative logos using the binding site sequences (given in bold type) of the corresponding species (Figure 5B).

A

C. albicans

Seq: C4_03520C_RBT1 TF: Efg1 Binding site: **ATGCATAA** Start position(s): [489] Total number: 1
Seq: C4_04320W_RBT2 TF: Efg1 Binding site: **ATGCATAA** Start position(s): [52] Total number: 1
RC_: C4_04320W_RBT2 TF: Efg1 Binding site: **ATGCATGT** Start position(s) rev_comp: [944] Total number: 1
Seq: C1_07030C_RBT4 TF: Efg1 Binding site: **ATGCATGT** Start position(s): [294] Total number: 1
RC_: C4_00120W_RBT6 TF: Efg1 Binding site: **ATGCATGA** Start position(s) rev_comp: [260] Total number: 1

C. parapsilosis

RC_: CPAR2_RBT2_401740 TF: Efg1 Binding site: **CTGCATA** Start position(s) rev_comp: [344] Total number: 1
Seq: CPAR2_RBT4_208800 TF: Efg1 Binding site: **CTGCATA** Start position(s): [360] Total number: 1

B

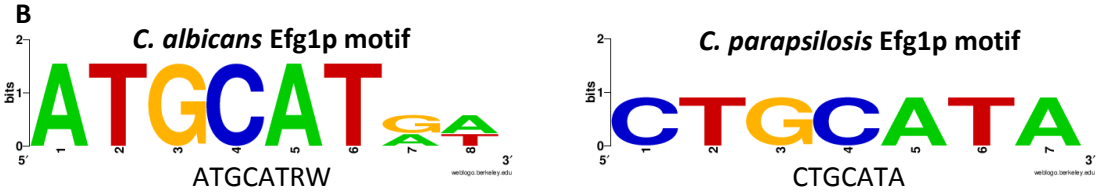

Figure 5| *C. albicans* and *C. parapsilosis RBT* gene promoter analyses in the TFbsST database. A| Analytical output of Efg1p binding sites in *C. albicans* and *C. parapsilosis RBT* family genes. **B|** Efg1p binding site motifs in *C. albicans* and *C. parapsilosis RBT* family

genes. Logos of the Efg1p binding site sequences were produced using free online software Weblogo (http://weblogo.berkeley.edu/logo.cgi).

Figure 6 is a graphical illustration of Efg1p localization in the promoters of *C. albicans* and *C. parapsilosis RBT* family genes based on its binding sites (Figure 6). Both, *C. albicans* and *C. parapsilosis* possessed Efg1p binding sites in *RBT2* and *RBT4* promoters. Efg1p had similar localization across the *RBT4* promoters in both *Candida* species. *C. albicans* Efg1p displayed overlapping motifs in the *RBT2* promoter. These findings support both our approach and database.

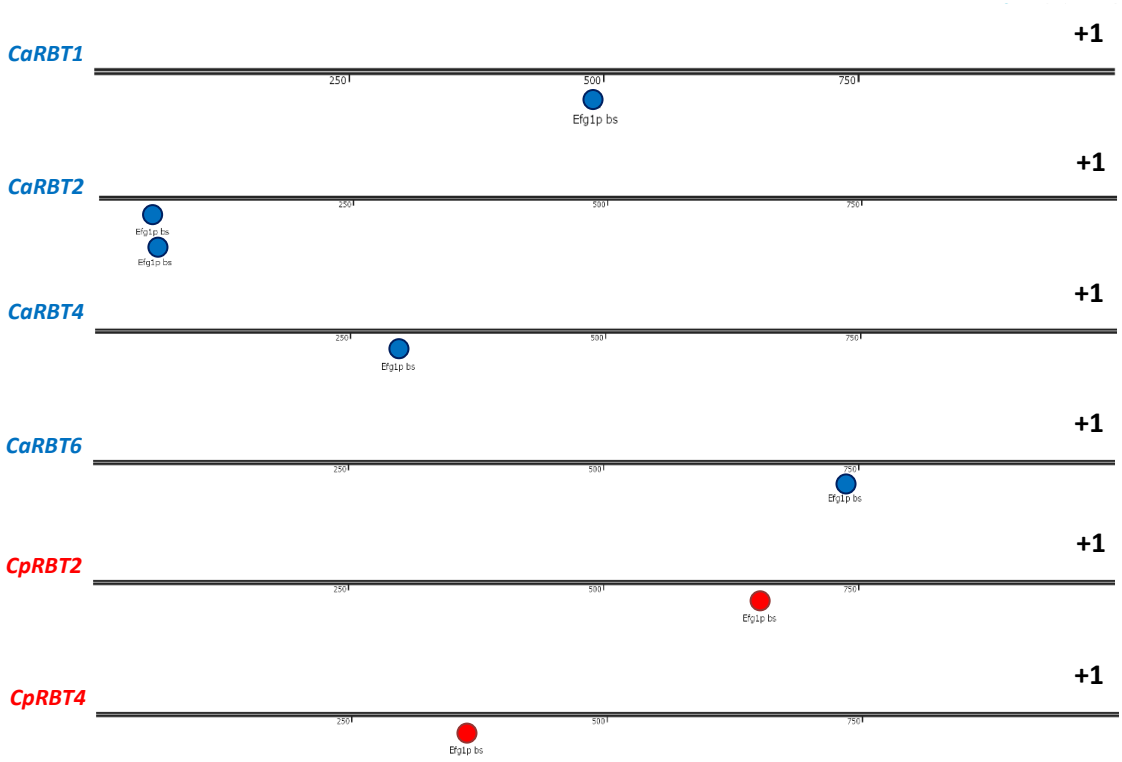

Figure 6| Efg1p binding sites across the *C. albicans* and *C. parapsilosis RBT* family gene promoters. Four *C. albicans RBT* family genes, *CaRBT1*, *CaRBT2*, *CaRBT4* and *CaRBT6* posses Efg1p binding sites with *CaRBT2* having overlapping sequences. Two *C. parapsilosis RBT* family genes, *CpRBT2* and *CpRBT4* posses Efg1p binding motifs. Efg1p has similar localization in *RBT4* promoters of both *Candida* species. +1 indicates an ORF starting point.

4.4 Discussion

C. albicans biofilms cause serious infections to nosocomial patients in hospitals. *C. albicans* biofilm-related genes are governed by 9 transcriptional regulators (Nobile *et al*. 2012; Fox *et al*. 2015). According to the DBD transcription factor prediction database, *C. albicans* possesses more than 500 putative TFs (www.transcriptionfactor.org). However, the binding site motifs of many TFs are unknown. Additionally, a limited number of bioinformatics tools for the analyses of *C. albicans* promoter sequences makes the *in silico* investigation even harder. Due to the latter, a novel bioinformatics tool, TFbsST database, was established to compile *C. albicans* TF binding site data. TFbsST software can be used to screen *Candida* gene promoter sequences for these TFs.

The vast majority of yeast databases are generated for a model yeast *S. cerevisiae* (UniPROBE, YEASTRACT, MYBS, YPA, JASPAR, TRANSFAC® and YeTFaSCo). To design the TFbsST application these databases were carefully reviewed and YeTFaSCo was used as the basis of our database. Nevertheless, there are significant differences between the YeTFaSCo and TFbsST databases. The main difference is that TFbsST includes TF motifs for *C. albicans*. Another important feature of the TFbsST database is that it also contains other *Candida* species such as *C. parapsilosis, C. dubliniensis* and *C. glabrata*. Additionally, the TFbsST database can perform TF frequency analysis (outputs a table with %) along with the TF localization analysis (outputs graphs and tables). Therefore, TFbsST is a user-friendly and dynamic website that can be used for more complete gene regulation analyses in *Candida*.

However, a list of rational criteria for further evaluation of the motifs and database tools was generated to optimise the TFbsST database. To improve *in silico* prediction power a sophisticated TF motif scoring system based on their establishment method (e.g. ChIP-chip or gene expression) is planned to be incorporated in the TFbsST database. In addition, GO annotation and inter-study agreement could provide further details and relevant scores to the TF binding sites. Clearly, browsing TFbsST reveals that limited piece of information is available for the *Candida* species other than *C. albicans*. Due to the latter, easily modifiable text files were integrated into the TFbsST website. This flexible structure facilitates rapid enrichment of TF, motif and related organism lists.

The TFbsST database was used to analyse TF frequencies in *C. albicans* genome. These *in silico* analyses showed several TF motifs that were ubiquitous in nearly all *C. albicans* genes (Hcm1p, Srr1p and Yox1p) and others that were significantly rare (Tac1p, Rob1p, Rpn4p,

Mcm1p and Mbp1p). Hcm1p is a forkhead TF that plays an important role in fungal morphogenesis (Bensen *et al*. 2002) and iron homeostasis (Singh *et al*. 2011). Srr1p is a 2-component response regulator also required for morphogenesis (Desai *et al*. 2011) and H_2O_2 (hydrogen peroxide) resistance (Bruce *et al*. 2011). Yox1p is a putative transcriptional repressor (Tuch *et al*. 2008) that peaks at G1/S phase of cell cycle (Cote *et al*. 2009). Despite their important role, it is unlikely that these TFs regulate the expression of all genes in *C. albicans*. Thus, more research is required to reveal the most accurate DNA binding site motifs of these TFs. Closer look at some TFs predicted to regulate small number of genes revealed that these TFs are parts of a larger protein complex. For example, Mbp1p and Tac1p represent part of MBF complex (Cote *et al*. 2009) and DRE (drug responsive element) element respectively. Other TFs in this group regulate different functions in the cell. For instance, Rob1p regulates biofilm development (Liu *et al*. 2005; Nett *et al*. 2009) and a C_2H_2 (Acetylene) TF, Rpn4p, regulates proteasome synthesis (Gasch *et al*. 2004; Enjalbert *et al*. 2006). Mcm1p is a protein with unknown function (CGD). This data suggest that more research is required to elucidate the exact role of these TFs.

Mcm1p and Mbp1p were predicted to be overrepresented in *RBT* family genes. However, a closer inspection of Mcm1p and Mbp1p in the *RBT* family indicated that binding site motifs of these TFs were present in a single *RBT* gene. Overrepresentation was due to their low frequency in *C. albicans* genome. The remaining overrepresented TFs in *RBT* genes, predicted on the basis of their binding sites, regulate biofilm formation in *C. albicans*. Bcr1p, Efg1p, Ndt80p are master regulators (Nobile *et al*. 2012), Cph2p regulates filamentous growth (Lane *et al*. 2001) and Cwt1p governs cell wall integrity (Moreno *et al*. 2003). Efg1p and Rpn4p were overrepresented in the *ALS* gene family. However, binding sites of Rpn4p were present only in a single *ALS* gene (*ALS9*). Efg1p though was predicted to be present in some genes of both gene families, *RBT* and *ALS*. These results suggest that Efg1p can regulate important gene families that shape biofilm development in *C. albicans*. Efg1p binding sites were also found in the *C. parapsilosis RBT* family genes. This finding suggests that Efg1p can regulate *RBT* genes across different species of *Candida*. To experimentally validate these *in silico* analyses, gene expression experiments of *Candida EFG1* mutants grown in YNBNP (filament-inducing medium) can be conducted quantifying *RBT* genes expression with RTqPCR.

To analyse the effect of bacterial supernatants on *Candida*, differentially expressed genes of *C. albicans*, defined by Holcombe *et al*. (2010) that were significantly upregulated or

downregulated in response to *P. aeruginosa* supernatants, were annotated with Blast2Go. The up-to-date annotation of these genes elucidated the localization of many proteins that were not previously annotated. These data provide new insights about the proteins that are involved in *Candida – Pseudomonas* interactions. The question now is whether these genes are regulated by a specific transcription regulator or if they are governed by different TFs.

4.5 Appendix

Table 6| Gene ontology (GO) annotations of upregulated gene set in response to the supernatants derived from 4 strains of *Pseudomonas*, PAO1, CF144, CF177 and ΔQS. This Holcombe *et al*. (2010) dataset was re-analysed here and up-to-date GO annotations were obtained with Blast2Go. This table is summarised in the Figure 3A (green bars).

ID	Gene	GO – cellular component
Plasma membrane		
C4_01100C	*AGP2*	Integral component of plasma membrane
C3_03070W	***AMF1***	**Integral component of membrane**
C3_04070C	*CDR11*	Integral component of membrane
C2_06020W	*CNT*	Integral component of plasma membrane
CR_09370W	*ELF1*	Plasma membrane
C7_02910W	*ENA21*	Integral component of membrane
C4_03700W	***FNX1***	**Integral component of membrane**
C4_05430C	*GAP5*	Integral component of plasma membrane
C6_00330C	*GNP1*	Integral component of plasma membrane
C4_06760W	*GUT2*	Plasma membrane
C6_03790C	*HGT10*	Integral component of plasma membrane
C1_13130C	*HIP1*	Integral component of plasma membrane
C1_09680W	***MTS1***	**Plasma membrane**
C4_01940W	*PHO89*	Integral component of plasma membrane
C1_09210C	*SGE11*	Integral component of membrane
C6_03840C	*SNQ2*	Plasma membrane; Integral component of membrane
C2_09900C	*TIM23*	Plasma membrane
CR_04200W	*YDJ1*	TRC complex
C2_08590W	*YWP1*	Anchored component of membrane
Cytoplasm		
C1_07710C	*ADE4*	Cytoplasm
C2_01270W	*CHA1*	Cytoplasm
C5_03640W	*DPH51*	Cytosol
C2_05100C	*ERF1*	Cytoplasmic stress granule; Cytosol
C6_02500C	*GCV1*	Cytoplasm
C1_08400C	*GCV2*	Cytosol
C1_10450W	*GLY1*	Cytosol
C1_09490C	*GUA1*	Cytoplasm
C2_06390C	*IMH3*	Cytoplasm
C6_02230W	*LSG1*	Cytosolic large ribosomal subunit
C4_04720W	*MTD1*	Cytosol
CR_06720W	*NMD3*	Cytosolic large ribosomal subunit
C5_00260W	*PRS1*	Cytosol
C1_03350C	*RLI1*	Cytosolic ribosome
C1_11040W	*RPL29*	Cytosolic large ribosomal subunit
C1_06890C	***RPL34B***	**Cytosolic large ribosomal subunit**
C1_11360W	*RPL37B*	Cytosolic large ribosomal subunit
C3_04680W	*RPP2B*	Cytosol
C6_00650C	*RPS13*	Cytosolic small ribosomal subunit
C3_04670C	*RPS15*	Cytosolic small ribosomal subunit
C1_01370C	*RPS21B*	Cytosolic small ribosomal subunit
C3_00090W	*RPS24*	Cytosolic small ribosomal subunit
CR_07630C	*RPS27*	Cytosol
C7_00710W	*RPS28B*	Cytosolic small ribosomal subunit
C3_04860W	*SFP1*	Cytoplasm
C2_03220C	*STP4*	Cytoplasm
C5_02490C	*TIF5*	Cytosolic small ribosomal subunit
C5_04570C	*URA7*	Cytoplasm
CR_04200W	*YDJ1*	Perinuclear region of cytoplasm

Nucleus		
C3_05160C	*DBP10*	Nucleolus
CR_02530W	***DBP2***	**Nucleus**
C1_10030W	*DBP3*	Nucleolus
C5_03640W	*DPH51*	Nucleus
CR_09370W	*ELF1*	Nucleus
C1_04130W	***ERB1***	**Nucleoplasm; PeBoW complex**
C2_05100C	*ERF1*	Nucleus
C3_06850W	*FCR1*	Nucleus
C5_04750C	*HAS1*	Nucleolus; Nuclear envelope
C2_08000C	*KRE30*	Nucleus; Nucleoid
C6_02770W	*MRT4*	Nucleoplasm; Nucleolus
C4_03030C	*NAN1*	rDNA heterochromatin; RENT complex; t-UTP complex
C2_00140W	*NDT80*	Nuclear chromatin
CR_05520W	***NOC2***	**Nucleolus; Noc1p-Noc2p complex; Noc2p-Noc3p complex**
C6_03640W	***NOG2***	**Nucleolus; Nucleoplasm**
C4_06720W	*NOP1*	Nuclear chromosome; Cajal body
C7_04230W	*NRG1*	Nucleus
C6_00920W	**orf19.93**	**Nucleus**
C5_00260W	*PRS1*	Nucleus
C2_07450C	*RCL1*	Nucleolus
C1_03350C	*RLI1*	Nucleus
C7_00570W	*RPA135*	DNA-directed RNA polymerase I complex
C3_00090W	*RPS24*	Nucleolus
C1_12680W	***RRB1***	**Nucleolus**
C2_08480W	*RRP8*	Nucleolus
C3_04860W	*SFP1*	Nucleus
C3_04380C	*SNU13*	Spliceosomal complex; U4/U6 x U5 tri-snRNP complex
C2_03220C	*STP4*	Nucleus
C1_02790W	*TIF34*	Eukaryotic translation initiation factor 3 complex
C3_02130W	*UTP4*	t-UTP complex
Ribosome		
C3_05160C	*DBP10*	Preribosome, large subunit precursor
C1_10030W	*DBP3*	Preribosome, large subunit precursor
C2_08490W	*DSE1*	Pwp2p-containing subcomplex of 90S preribosome
C1_04130W	*ERB1*	Preribosome, large subunit precursor
C5_04750C	*HAS1*	Preribosome, large subunit precursor
C2_08000C	*KRE30*	Ribosome
C6_02770W	***MRT4***	**Ribosome; Preribosome, large subunit precursor**
C4_03030C	*NAN1*	90S preribosome
CR_04360C	***NHP2***	**Ribosome**
C6_03640W	*NOG2*	Preribosome, large subunit precursor
C4_06720W	*NOP1*	Ribosome; 90S preribosome
C6_00370C	*NOP5*	90S preribosome
C5_00260W	*PRS1*	Ribose phosphate diphosphokinase complex
C1_03350C	*RLI1*	Preribosome, large subunit precursor
C2_06810C	*RPL11*	Ribosome
C6_02070C	*RPL23A*	Ribosome
C4_04900W	*RPL30*	Ribosome
C5_04590C	*RPL43A*	Ribosome
C3_04680W	*RPP2B*	Ribosome
C2_08040C	*RPS10*	Ribosome
C6_00650C	*RPS13*	90S preribosome
C7_00960W	*RPS18*	Small ribosomal subunit
C1_06460C	*RPS22A*	Ribosome
CR_07630C	*RPS27*	Ribosome
CR_09950C	*SIK1*	90S preribosome
C1_02790W	*TIF34*	Multi-eIF complex
C5_02490C	*TIF5*	Multi-eIF complex
C3_02130W	*UTP4*	90S preribosome
C3_05160C	*DBP10*	Preribosome, large subunit precursor
Mitochondrion		
C2_08100W	*CPD1*	Mitochondrion

CR_02530W	*DBP2*	Mitochondrion
CR_09370W	*ELF1*	Mitochondrion
C1_08400C	*GCV2*	Mitochondrion; Glycine cleavage complex
C4_06760W	*GUT2*	Integral component of mitochondrial outer membrane; Glycerol-3-phosphate dehydrogenase complex
CR_05520W	*NOC2*	Mitochondrion
C6_00920W	orf19.93	Mitochondrial intermembrane space
C3_00090W	*RPS24*	Mitochondrion
C2_09900C	*TIM23*	Mitochondrial inner membrane presequence translocase complex; Integral component of mitochondrial inner membrane
Cell wall/surface		
CR_10110W	*CHT3*	Cell surface; Extracellular region
C2_08490W	*DSE1*	Fungal-type cell wall
C5_02080C	*HSP12*	Yeast-form cell wall; Hyphal cell wall
C4_06720W	*NOP1*	Cell surface
C2_08870C	*PIR1*	Yeast-form cell wall; Extracellular region
C6_02070C	*RPL23A*	Hyphal cell wall
C2_08040C	*RPS10*	Cell surface
C6_00650C	*RPS13*	Hyphal cell wall
C5_04110W	*SCW11*	Cell surface; Fungal-type cell wall; Extracellular region
C2_08590W	*YWP1*	Cell surface; Yeast-form cell wall; Hyphal cell wall; Extracellular region
43S/48S preinitiation complex		
C5_02490C	*TIF5*	Eukaryotic 48S preinitiation complex
C2_10710W	*TIF11*	Eukaryotic 43S preinitiation complex; Eukaryotic 48S preinitiation complex
C1_02790W	*TIF34*	Eukaryotic 43S preinitiation complex; Eukaryotic 48S preinitiation complex
Small-subunit processome		
C2_08490W	*DSE1*	Small-subunit processome
C4_03030C	*NAN1*	Small-subunit processome
C4_06720W	*NOP1*	Small-subunit processome
C6_00370C	*NOP5*	Small-subunit processome
CR_09950C	*SIK1*	Small-subunit processome
C3_04380C	*SNU13*	Small-subunit processome
C3_02130W	*UTP4*	Small-subunit processome
Box C/D snoRNP complex		
CR_04360C	*NHP2*	Box H/ACA snoRNP complex
C4_06720W	*NOP1*	Box C/D snoRNP complex
C6_00370C	*NOP5*	Box C/D snoRNP complex
CR_09950C	*SIK1*	Box C/D snoRNP complex
C3_04380C	*SNU13*	Box C/D snoRNP complex
Vacuole		
C4_01100C	*AGP2*	Fungal-type vacuole membrane
C4_05430C	*GAP5*	Vacuole
C6_00330C	*GNP1*	Vacuole
C1_13130C	*HIP1*	Vacuole
Viral nucleocapsid		
CR_04360C	*NHP2*	Viral nucleocapsid
C3_04380C	*SNU13*	Viral nucleocapsid
CR_04200W	*YDJ1*	Viral envelope
Golgi apparatus		
C2_08100W	*CPD1*	Golgi apparatus
C6_02230W	***LSG1***	**Golgi apparatus**
Endoplasmic reticulum		
C4_01100C	*AGP2*	Endoplasmic reticulum membrane
C6_02230W	*LSG1*	Endoplasmic reticulum
Polysome		
CR_09370W	*ELF1*	Polysome
Intracellular region		
C1_02840W	*PDE2*	Intracellular region
Mating projection tip		
C6_02230W	*LSG1*	Mating projection tip

Not yet annotated to any cellular component	
C2_03520C	*ADAEC*
C6_02480W	*ADH7*
CR_06860C	*ARO10*
C1_10740C	*ASR1*
C3_07280C	*ENT4*
C2_10360C	*HEM3*
C4_02050W	*HGH1*
C4_02440C	*PGA38*
C1_07330W	*RME1*
C4_06390W	*SOU1*
C4_03370C	orf19.3364
C3_03460C	orf19.344
C2_05160C	orf19.3547
C5_01550C	orf19.4149
C1_10360C	orf19.4907
C1_11990W	orf19.5267
C7_01430C	orf19.6586

Up-to-date annotation of genes coding for proteins with previously unknown function is given in **bold** type (This study).

Table 7| Gene ontology (GO) annotations of the downregulated gene set in response to the supernatants derived from 4 strains of *Pseudomonas*, PAO1, CF144, CF177 and ΔQS. This Holcombe *et al*. (2010) dataset was re-analysed here and up-to-date GO annotations were obtained with Blast2Go. This table is summarised in the Figure 3A (red bars).

ID	Gene	GO – cellular component
Plasma membrane		
CR_07070C	*ALS3*	Plasma membrane; Anchored component of membrane
CR_02910W	***APG13***	**Extrinsic component of membrane**
C6_04210C	*ATM1*	Integral component of membrane
C1_05700W	*AUT7*	Extrinsic component of membrane
C1_06520C	***BPH1***	**Extrinsic component of membrane**
C2_03320W	*CHK1*	Membrane
C5_02460C	*ECM331*	Plasma membrane; Anchored component of membrane
C1_08590C	*ERG1*	Plasma membrane; Integral component of membrane; Lipid particle
C6_00440C	*FET34*	Plasma membrane
C1_14130W	*FTR1*	High-affinity iron permease complex
C3_05580C	*GAP2*	Integral component of plasma membrane
C7_00280W	*HGT12*	Integral component of plasma membrane
C1_02110C	*HGT2*	Integral component of plasma membrane
C2_04940C	*ITR1*	Integral component of plasma membrane
C2_07580W	*MAE1*	Integral component of membrane
C5_04930C	*MAL31*	Integral component of plasma membrane
C4_00430W	*MEP2*	Integral component of plasma membrane
C3_00650W	*NGT1*	Plasma membrane; Integral component of membrane
CR_02240C	*OPT2*	Plasma membrane
CR_02900W	*ScOPT2*	Plasma membrane
CR_02490W	*OPT4*	Plasma membrane
C4_03520C	*RBT1*	Anchored component of membrane
CR_06660W	*SEO1*	Integral component of membrane
C2_00680C	*SOD5*	Anchored component of membrane
C2_06010W	***SPO72***	**Extrinsic component of membrane**
C2_02860W	*SUR2*	Integral component of membrane
C5_03060C	*TNA1*	Plasma membrane; Integral component of membrane
C3_06710W	***VHC1***	**Integral component of vacuolar membrane**
CR_03270W	*VHT1*	Integral component of plasma membrane
Cytoplasm		
C4_02410C	*AHP1*	Cytosol
C4_05560C	*ARO9*	Cytoplasm
C1_05700W	*AUT7*	Cytosol
CR_10360C	*CTM1*	Cytosol
C1_04660W	*DUR1,2*	Cytoplasm
C6_03340C	*GLC3*	Cytoplasm
C4_02990C	***GST2***	**Cytoplasm**
CR_10100C	*INO1*	Cytoplasm
C1_12010C	*KIP4*	Cytoplasm
CR_04480C	*LAP3*	Cytoplasm
C7_00400W	*LEU2*	Cytosol
C4_05320W	*LYS1*	Cytoplasm
C3_06590W	*LYS9*	Cytosol
CR_10790W	*MAL2*	Cytoplasm
C3_02950C	*MET13*	Cytosol
C4_00200C	*MET15*	Cytoplasm
C4_00150C	***PEX5***	**Cytosol**
CR_02820W	*PGM2*	Cytosol
C2_07570W	*RNR22*	Cytosol; Ribonucleoside-diphosphate reductase complex
C2_00680C	*SOD5*	Cytoplasm
CR_05720W	*TPS1*	Alpha,alpha-trehalose-phosphate synthase complex (UDP-forming)
CR_09010C	***YHR112C***	**Cytoplasm**

Nucleus		
C4_05560C	*ARO9*	Nucleus
C1_05700W	***AUT7***	**Nucleus**
C2_09940W	*GCN4*	Nucleoplasm; Nuclear chromatin; Transcription factor complex
C2_06650C	***PRP31***	**U4/U6 x U5 tri-snRNP complex**
C4_02030W	***RFX2***	**Nucleus**
C5_00980W	***TRY3***	**Nucleus**
C1_06280C	*UME6*	Nucleus
CR_09930W	***YDR124W***	**Nucleus**
CR_09010C	***YHR112C***	**Nucleus**
Cell wall/surface		
C4_02410C	*AHP1*	Yeast-form cell wall; Hyphal cell wall
CR_07070C	*ALS3*	Yeast-form cell wall; Hyphal cell wall; Cell surface
C4_03470C	*ECE1*	Hyphal cell wall
C5_02460C	*ECM331*	Fungal-type cell wall; Cell surface
C6_00440C	*FET34*	Cell surface
C1_14130W	*FTR1*	Cell surface
CR_10100C	*INO1*	Hyphal cell wall; Cell surface
C3_01360C	***IRO1***	**Cell**
C4_00200C	*MET15*	Hyphal cell wall
C4_03520C	*RBT1*	Cell wall
C2_00680C	*SOD5*	Yeast-form cell wall; Hyphal cell wall; Cell surface
Extracellular region		
CR_07070C	*ALS3*	Extracellular region
C5_02460C	*ECM331*	Extracellular region
CR_10790W	*MAL2*	Extracellular region
C4_03520C	*RBT1*	Extracellular region
C1_07030C	*RBT4*	Extracellular region
C2_00680C	*SOD5*	Extracellular region
C7_00260C	***YLR001C***	**Extracellular space**
Mitochondrion		
C6_04210C	*ATM1*	Mitochondrial inner membrane
C4_04620C	***CTN2***	**Mitochondrion**
CR_01400W	***LYS12***	**Mitochondrion**
C3_02950C	*MET13*	Mitochondrion
C2_08390W	orf19.1433	Mitochondrion
C7_00260C	***YLR001C***	**Mitochondrion**
Vacuole		
C4_02360W	*AMS1*	Fungal-type vacuole membrane
C1_05700W	*AUT7*	Fungal-type vacuole membrane
C6_00440C	*FET34*	Fungal-type vacuole membrane
C2_06880C	*PRB1*	Fungal-type vacuole
C7_00260C	***YLR001C***	**Fungal-type vacuole membrane**
Endoplasmic reticulum		
C1_06520C	*BPH1*	Endomembrane system
C1_08590C	*ERG1*	Endoplasmic reticulum membrane
C4_06810C	***SLY1***	**Endoplasmic reticulum; ER to Golgi transport vesicle**
C2_02860W	*SUR2*	Endoplasmic reticulum membrane
Intracellular		
C1_06520C	*BPH1*	Intracellular
C4_06480C	*CEK1*	Intracellular
C2_03320W	*CHK1*	Intracellular
C5_00450C	***IFG3***	**Intracellular**
CR_07190W	***RGD2***	**Intracellular**
Autophagosome		
CR_02910W	*APG13*	Pre-autophagosomal structure membrane
C1_05700W	*AUT7*	Autophagosome membrane; Pre-autophagosom; Cvt vesicle membrane
C2_06010W	***SPO72***	**Pre-autophagosomal structure**
Peroxisome		
C4_04620C	*CTN2*	Peroxisome
C3_05360C	***LON1***	**Peroxisomal matrix**
C4_00150C	*PEX5*	Peroxisomal membrane

Golgi apparatus		
C4_00430W	*MEP2*	Golgi apparatus
C4_06810C	*SLY1*	Golgi membrane
Microtubule		
C1_12010C	*KIP4*	Microtubule; Kinesin complex
Vacuole		
C3_05580C	*GAP2*	Vacuole
Viral nucleocapsid		
C2_06650C	*PRP31*	Viral nucleocapsid
Endosome		
C2_06010W	*SPO72*	Late endosome
Glyoxysome		
C1_04500W	*ICL1*	Glyoxysome
Not yet annotated to any cellular component		
CR_02070C	*ADH5*	
C4_06340W	*AGO1*	
C2_03120W	*AMO1*	
C3_01820W	*BGL98*	
CR_01930C	*BIO2*	
C2_02950W	*BNA3*	
C1_08170C	*BUL1*	
C5_02690W	*BZD99*	
C6_01070C	*CIP1*	
C3_04550C	*CMK1*	
C2_00690W	*EEP2*	
C4_03910W	*FGR28*	
C6_01650C	*FMP27*	
C3_06450W	*GLG2*	
C1_01360C	*GLG21*	
C6_00840W	*GPX2*	
C1_07520C	*IST2*	
C1_00170W	*LEU4*	
C6_03310W	*LPF39*	
C7_03470W	*LPF44*	
C1_02820W	*LYS2*	
C2_04460W	*LYS22*	
CR_07220C	*NDL1*	
C5_05190W	*PCL5*	
C4_01850C	*PDC12*	
C1_08950W	*PFK26*	
C6_03320W	*PHZ1*	
C1_13160W	*PSA2*	
C4_03940C	*PYC2*	
C6_03260W	*RNH11*	
C5_03930C	*SIA1*	
C4_00190W	*SMA2*	
C2_10690W	*TPS3*	
C7_00170W	*VPS70*	
CR_10570C	*YHB4*	
C1_01930W	*YIL024C*	
C2_02390W	orf19.1562	
C3_02330C	orf19.1611	
C6_04480C	orf19.2132	
C3_01020W	orf19.2506	
C1_02730W	orf19.2962	
C1_05440C	orf19.419	
C2_04400W	orf19.4513	
C4_01860C	orf19.4607	
C1_09310C	orf19.4791	
C1_09340C	orf19.4795	
C1_10060C	orf19.4873	
C1_13430C	orf19.4970	

C7_03280C	orf19.5125
CR_06570C	orf19.915

Annotation of genes coding for proteins with previously unknown function is given in **bold** type (This study).

Table 8| Gene ontology (GO) annotations of the upregulated gene set in response to the HSL-containing *Pseudomonas* supernatants. This Holcombe *et al*. (2010) dataset was re-analysed here and up-to-date GO annotations were obtained with Blast2Go. This table is summarised in the Figure 3B (green bars).

ID	Gene	GO – cellular component
Plasma membrane		
C6_03700W	*ALS1*	Plasma membrane; Anchored component of membrane
C1_09150W	*AOX2*	Plasma membrane; Integral component of membrane
C4_04310W	*COX11*	Plasma membrane
C4_01160W	*CRD2*	Integral component of membrane
C2_02280W	*FMP39*	Plasma membrane; Integral component of membrane
C5_02790C	*GAP1*	Integral component of plasma membrane
C5_03500W	*GAP6*	Integral component of plasma membrane
C2_02610C	*HGT20*	Integral component of plasma membrane
C3_02310W	*MEP1*	Integral component of plasma membrane
C1_11870W	*MUP1*	Integral component of plasma membrane
CM_00310W	*NAD2*	Integral component of membrane
C5_03800W	**orf19.1114**	**Plasma membrane; Integral component of mitochondrial inner membrane**
C1_08610C	**orf19.409**	**Plasma membrane; Integral component of membrane**
C4_04080C	*PGA31*	Anchored component of membrane
C3_03800W	*PTR22*	Plasma membrane; Integral component of membrane
C4_02890C	*QCR9*	Plasma membrane
C2_06470W	*RTA2*	Plasma membrane; Integral component of membrane; Membrane raft
C2_06460W	*RTA3*	Integral component of membrane
CR_09170C	*SSU1*	Plasma membrane; Integral component of membrane
Mitochondrion		
C1_09150W	*AOX2*	Mitochondrion; Respiratory chain
C7_03380W	*CMC2*	Mitochondrial intermembrane space
C4_04310W	*COX11*	Mitochondrial inner membrane; Mitochondrial intermembrane space; Mitochondrial ribosome
C2_01180W	*COX17*	Mitochondrial intermembrane space
C2_02280W	*FMP39*	Mitochondrial membrane
C2_10240W	*GPD1*	Glycerol-3-phosphate dehydrogenase complex
C6_02010C	*GPD2*	Glycerol-3-phosphate dehydrogenase complex
CM_00310W	*NAD2*	Mitochondrial respiratory chain complex I
C5_03800W	**orf19.1114**	**Mitochondrial respiratory chain supercomplex**
C4_02890C	*QCR9*	Mitochondrial respiratory chain complex III
Cell wall/surface		
C6_03700W	*ALS1*	Yeast-form cell wall; Hyphal cell wall; Cell surface
C4_01160W	*CRD2*	Yeast-form cell wall
C5_02790C	*GAP1*	Fungal-type cell wall; Cell surface
C6_02010C	*GPD2*	Cell surface
C4_04080C	*PGA31*	Yeast-form cell wall; Cell surface
Cytoplasm		
CR_06950C	*ATX1*	Cytosol
CR_02330C	***KAR4***	**Cytoplasm**
C3_07430W	**orf19.6747**	**Cytosol**
C7_02810W	*PRX1*	Cytoplasm
Nucleus		
CR_06950C	*ATX1*	Nucleus
C7_02810W	*PRX1*	Nucleus
C7_03380W	**orf19.1336.2**	**Nucleus**
Vacuole		
C5_02790C	*GAP1*	Vacuole
C5_03500W	*GAP6*	Vacuole
Extracellular region		

C6_03700W	*ALS1*	Extracellular region
C4_04080C	*PGA31*	Extracellular region
Exocyst		
C4_04310W	*COX11*	Exocyst
Not yet assigned to any cellular component		
C6_02480W	*ADH7*	
C2_00340C	*ARO8*	
C1_04450C	*FMA1*	
C1_02980W	*GOR1*	
C2_02940W	*MET1*	
C6_01420C	*OYE23*	
CR_02580W	*PAN6*	
C1_05770C	*PRC3*	
C3_00320W	*RHR2*	
C2_02900W	orf19.5814	

Up-to-date annotation of genes coding for proteins with previously unknown function is given in **bold** type (This study).

Table 9| Gene ontology (GO) annotations of the downregulated gene set in response to the HSL-containing *Pseudomonas* supernatants. This Holcombe *et al*. (2010) dataset was re-analysed here and up-to-date GO annotations were obtained with Blast2Go. This table is summarised in the Figure 3B (red bars).

ID	Gene	GO – cellular component
Plasma membrane		
C3_00920W	*ATO1*	Integral component of membrane
C6_00790C	*CTR1*	Plasma membrane; Integral component of membrane
CR_07290W	*FRE7*	Integral component of membrane
CR_02210W	***FRK1***	**Cell cortex**
C1_01980W	*HGT1*	Integral component of plasma membrane
C4_01070W	*HGT17*	Integral component of plasma membrane
C4_03570W	*HWP1*	Anchored component of membrane
C4_04030W	*JEN2*	Integral component of plasma membrane
CR_01220W	*TNA12*	Integral component of membrane
Nucleus		
CR_09880W	*DEF1*	Nucleus
C1_04040C	***NOP9***	**Nucleolus**
CR_00310C	*RNT1*	Nucleus
CR_03890W	***WOR3***	**Nucleus**
C3_02640C	*ZCF1*	Nucleus
Ribosome		
C1_04040C	*NOP9*	90S preribosome; Preribosome, small subunit precursor
α DNApol:primase complex		
C1_07490C	***POL12***	**Alpha DNA polymerase:primase complex**
Cell wall/surface		
C4_03570W	*HWP1*	Cell wall
C2_01380W	*PLB4.5*	Cell surface
Bud		
C2_05260W	***BUD14***	**Cellular bud neck; Cellular bud tip; Incipient cellular bud site**
Cytoplasm		
C1_02120C	*SHA3*	Cytoplasm
Exosome		
C2_08550C	*MSU1*	Exosome (RNase complex)
Fatty acid synthase complex		
C3_04830C	*FAS2*	Fatty acid synthase complex
Intracellular part		
CR_10340W	*PTP3*	Intracellular part
Mitochondrion		
C3_04830C	*FAS2*	Mitochondrion
Trans-Golgi network		
C7_03480W	*YEL1*	Trans-Golgi network
Not yet annotated to any cellular component		
CR_00640W	*ACC1*	
C4_00960W	*PTC8*	
C6_02330W	orf19.3475	
C1_00190C	orf19.6084	

Up-to-date annotation of genes coding for proteins with previously unknown function is given in **bold** type (This study).

Chapter 5

General Discussion

5. General discussion

5.1 Introduction

In the final part, the main findings of this research are briefly summarised and integrated with the existing knowledge to describe the main conclusions. A detailed experimental plan and database update strategies are outlined for the progression of this project. Also, new insights in *C. albicans* biofilms and interspecies interaction are highlighted indicating future directions of the field. This chapter concludes with the recommendation for investigation of *C. albicans* biofilms rather than of the planktonic cells, focus on the polymicrobial communities instead of monocultures and suggestion of translational studies from *in vitro* to *in vivo* models.

5.2 Research result summary

Protein kinases (PK) and transcription factors (TF) mediate signal transduction and transcription of proteins involved in *C. albicans* biofilm development. We described 5 additional PKs, *VPS15, PKH3, PGA43, IME2* and *CEX1*, that could contribute to the efficient filamentation and robust biofilm development. To identify the 'Poor' biofilm former mutants we used different biofilm and morphology assays from those who had previously screened this PK collection (Blankenship *et al*. 2010; Fanning *et al*. 2013; de Castro *et al*. 2013; Morales *et al*. 2013). Filamentation is essential for biofilm development but our screens highlighted additional processes, such as vascular biosynthesis and ribosome biogenesis, that were also important for *C. albicans* biofilms. The discovery that the individual elements of the MAPK pathway were not essential for *Candida* biofilms was unexpected suggesting that in order to filament the MAPK mutants may exploit the functionally overlapping MAPK pathways. However, consistent with earlier publications of Holcombe *et al*. (2010) and McAlester *et al*. (2008), *P. aeruginosa* supernatants were shown to have 2 distinct effects on *C. albicans*: HSL-independent biofilm impairment and HSL-dependent filamentation inhibition. To further investigate TF-regulated genes in *Candida* we created a TFbsST database and found that one of the master biofilm regulators, Efg1p, is also implicated in yeast-hyphae switch. Efg1p binding site elements were additionally predicted to be conserved in *C. parapsilosis* hyphae-related genes (*RBT*). Currently, the TFbsST database is limited to some *Candida* species TFs but these findings increase our knowledge of *Candida* biofilms and inter-species interactions adding novel bioinformatics tools to answer interesting questions of, for example, *Candida* gene regulation mechanisms.

5.3 *Candida albicans* interaction with bacteria

This research was focused on the investigation of *C. albicans – P. aeruginosa* interaction but *C. albicans* is also known to communicate with Gram-positive bacteria in addition to *P. aeruginosa*, which is Gram-negative, and increase the health burden. Polymicrobial infections are hard to treat since they include various pathogens such as bacteria, yeast and viruses, requiring different treatments. These pathogens possess synergistic or antagonistic behaviour affecting the dynamics of their communities and the outcome of the therapies. The *Candida*-bacterial interaction can promote or prevent the infections in the human-host. For example, a bacterium *Streptococcus gordonii* promotes the adherence of *C. albicans* cells, filamentous growth of hyphae and *C. albicans* biofilm formation on the epithelial cells of the oral cavity (reviewed by Morales and Hogan 2010). *S. gordonii* attaches to the epithelial cell surface with its polypeptides promoting the adherence of *C. albicans*. In return, the presence of *C. albicans* decreases the oxygen to the preferred levels by *S. gordonii*. This leads to the increased growth of *S. gordonii* communities (reviewed by Morales and Hogan 2010). These conditions in the oral cavity favour the development of yeast-bacterial biofilms that are difficult to treat with the existing therapies.

The synergetic behaviour of *C. albicans* and another Gram-positive bacterium, *Staphylococcus aureus*, also ultimately leads to robust biofilm development, significantly increasing mortality rates in mice (Shirtliff *et al*. 2009; Harriott and Noverr 2009). In these mixed biofilms, *C. albicans* prostaglandin E2 (hormone-like fatty acid) stimulates the growth of *S. aureus* (Krause *et al*. 2015). Urinary tract infections are enhanced by the cooperation of *C. albicans* and *Escherichia coli* (Gram-negative), that increases the attachment ability of *C. albicans* on the surface of the epithelial cells (Levison and Pitsakis 1987). The lipopolysaccharides of *E. coli* further enhance the mortality rates of *C. albicans*-infected mice (Akagawa *et al*. 1995).

However, the human host can benefit from the antagonistic behaviour of *C. albicans* and *Lactobacillus*. Different *Lactobacillus* species that are normal residents of the intestinal and female reproductive tracts can prevent adherence of *C. albicans* on the surface of the epithelial cells (reviewed by Morales and Hogan 2010). They secrete surlactins that prevent the adherence of *C. albicans* and compete for the attachment on the cell surface. These data reflect the complexity of mixed infection and the inter-kingdom interaction. Hence, more emphasis should be given to the studies of the polymicrobial communities.

5.4 *Pseudomonas aeruginosa* interaction with fungi

In this study we looked at the effects of *P. aeruginosa* on *C. albicans* but *P. aeruginosa* was also shown to affect other fungi. Conditions like CF and burn wounds involve polymicrobial infections with mixed bacterial and yeast communities. For example, the lungs of the CF patients are colonised by bacteria (mainly *P. aeruginosa*) and yeast including *C. albicans*, different *Aspergillus* and *Scedosporium* species and *Exophiala dermatitidis* (reviewed by Pihet *et al*. 2009). The study of the mixed cocultures indicated that *P. aeruginosa* can kill *Aspergillus fumigatus* conidia (Manavathu *et al*. 2014) and inhibit the growth of *Scedosporium aurantiacum* (Kaur *et al*. 2015). *P. aeruginosa* extracellular molecules can also inhibit *A. fumigatus* biofilm formation (Mowat *et al*. 2010) and its phenazines can manipulate *A. fumigatus* iron homeostasis (Briard *et al*. 2015). In contrast, *P. aeruginosa* volatiles stimulate the growth of *A. fumigatus* colonies on minimal media (Briard *et al*. 2016). The interaction between *P. aeruginosa* and *E. dermatitidis* (black yeast) remains unclear. These interactions highlight the importance of *in vivo* models that can shed more light to the mixed infections.

5.5 *In vivo* models for the investigation of fungal biofilms

We investigated the *C. albicans* biofilms and inter-kingdom cross-talk highlighting several aspects that are important for the biofilm formation *in vitro*. The majority of the researchers study fungal biofilms and yeast-bacterial interaction *in vitro*. The wealth of knowledge generated from the *in vitro* analyses can be employed to clarify the microbial interaction and biofilm development *in vivo*. Plethora of *in vivo* models is utilised for the investigation of fungal biofilms that significantly contribute to the knowledge of biofilm physiology and microbial interaction with the host. The *in vivo* biofilm development is significantly affected by the host factors including the host antibodies, nutrient availability, flow of the liquids (e.g. blood, urine) and the substrate properties. Although biofilms are known to be developed by several medically important fungi, *Aspergillus, Pneumocystis, Blastomyces, Zygomycetes, Trichosporon, Cryptococcus, Fusarium* and *Malassezia, Candida* biofilms are the mostly studied since *C. albicans* is a model pathogen for the investigation of the fungal biofilm infections (Reviewed by Nett and Andes 2016).

Many *in vivo* models are developed for the study of the *Candida* biofilm-related infections but the most popular is a central venous catheter model that is adopted for utilisation in a mouse (Lazzell *et al*. 2009), a rat (Andes *et al*. 2004) and a rabbit (Schinabeck *et al*. 2004). This model involves the insertion of a vascular catheter employing the surgical procedures

and allows the assessment of antifungal influence on the biofilm growth following systemic administration of a drug. These models significantly contributed into the establishment of the multi-drug resistance phenomenon *in vivo* directing the research towards the search for more effective therapies. Another, subcutaneous implant model in a mouse (Zumbuehl *et al*. 2007) and a rat (Ricicova *et al*. 2010), was designed using amphogel, hydrogel and amphotericin B-containing disks, inoculated with *C. albicans* and implanted subcuntaneously. Employing this model, Zumbuehl *et al*. (2007) showed that the hydrogel of this antifungal is suitable for the prevention of device-related infections due to its long-term efficiency.

To investigate denture infections that are common within the denture-wearers, a rat denture stomatitis model with a novel intraoral system was developed by Johnson *et al*. (2012). The previous *Macacairus* monkey models contributed into the knowledge of host response to denture biofilms but the less costly rat models were found to be more useful for the drug efficacy studies. Additional to the animal models, a human dental plaque model is used for more realistic studies of the oral communities. To study the oral flora, disks made of different materials are inserted in the removable intrabuccal splints of volunteers and after removal of the discs bearing oral microbial communities are analysed by the researchers (Rimondini *et al*. 1997; Scarano *et al*. 2004).

To study oropharyngeal and vaginal candidiasis the mouse oral (Dongari-Bagtzoglou *et al*. 2009) and vaginal mucosal models were developed respectively (Harriott *et al*. 2010). These models revealed the complexity of the oral biofilms that involved bacteria, neutrophils of host innate immune system (WBC) and keratin that protects epithelial cells from damage/stress (Dongari-Bagtzoglou *et al*. 2009). A vaginal candidiasis model however, showed that vaginal biofilms and those developed on abiotic surfaces share regulation factors (Harriott *et al*. 2010).

The clinical scenario of candiduria was characterised using a mouse urinary catheter model (Wang and Fries 2011). This model employs an insertion of a catheter with a guide wire into the bladder through the urethra of a female mouse. To investigate the candiduria and other catheter-related infections, this animal is infected with *C. albicans* intravesicularly. The occurred infection is rapidly detectable persisting up to a month and closely resembles the *Candida* biofilm formation environment in the patients (Wang and Fries 2011). Thus, this model can also be used for *C. albicans* biofilm-related studies in the future.

Clearly, animal models of biofilm-associated infections are beneficial for pathogenesis and drug discovery studies. However, more *in vivo* models are required to study the biofilms of other clinically relevant niches, for instance of (burn) wounds.

5.5.1 *In vivo* models for the investigation of bacterial biofilms

This project was mainly focused on fungal biofilms but bacterial biofilms also cause serious infections. Diverse *in vivo* models are developed to mimic the bacterial biofilm infections addressing important therapeutic questions of bacterial infections. *In vivo* biofilm infection models include the rat and mouse CF models for the investigation of the lung microbiome (Keiser and Engelhardt 2011), a murine cystitis (bladder infection) model for the study of bacteria inoculated in the bladder (Justice *et al*. 2004), an excisional wound model (mice) for the *S. aureus* infection in the cut wounds (Akiyama *et al*. 1996) and a rabbit model for the infective endocarditis where a high bacterial concentration is injected intravenously (Xiong *et al*. 2006).

In this research, fungal biofilms formed on abiotic surface were explored in great detail but bacterial biofilms can also be found on abiotic surfaces. The *in vivo* device-related infection models are common for the investigation of the bacterial biofilms developed on medical devises. The most popular are the rat vascular catheter models that were developed with the utilisation of the plastic catheter inserted in the vein of the rat (Ebert *et al*. 2011). Additionally, many urinary catheter models were described in mice, rats and rabbits in order to study the diverse aspects of the catheter-related infections. For example, using the urethral infection model Cirioni *et al*. (2011) examined the effects of the antibacterials on the infections caused by *P. aeruginosa* (Cirioni *et al*. 2011) and Allison *et al*. (2011) described the eradication of the bacterial persister cells via aminoglycoside therapy that inhibits protein synthesis (Allison *et al*. 2011). The orthopaedic and dental implant models also contribute into the understanding of the bacterial biofilms. To investigate the fungal biofilms, these models can be modified by incorporating the aspects of fungal infection and biofilms.

5.6 Future directions for the progression of this project

5.6.1 *Candida albicans* biofilm studies using 'Poor' biofilm former protein kinase mutants

Based on our discussion, the future research should be directed towards the detailed investigation of *C. albicans* biofilms in addition to its planktonic cells. The results of this Ph.D. project provide useful lead towards different directions for further research. Many questions arise in relation to the PKs that were firstly assigned to *C. albicans* biofilm

formation and filamentation in this study (Chapter 2). Hence, for the progression of this work, the mutants disrupted in these PK coding genes can be studied in detail. More specifically, 5 genes *VPS15, PKH3, PGA43, IME2* and *CEX1* disrupted in the mutants firstly associated with poor biofilm formation and impaired filamentous growth in this study can be followed up for further clarification of their role in *C. albicans* (Chapter 2). Vps15p, involved in vacuolar protein sorting and Pkh3p, required for PKC activity and cell-substrate adherence (Fanning *et al*. 2012), were clustered together in the protein-protein interaction networks (Chapter 2). To clarify their association, vacuolar morphology of the mutants disrupted in the genes coding for these proteins can be examined microscopically and their cell wall proteome can be determined with the spectrometry. Pga43p is probably required for cell wall integrity (Moreno-Ruiz *et al*. 2009) and can additionally be included in these studies. Apart from their role in *C. albicans* biofilm formation and filamentation, *IME2* and *CEX1* are not yet confidently assigned to any specific function. However, mutant disrupted in *IME2* is hypersensitive to amphotericin B (Xu *et al*. 2007) and *CEX1* is largely unexplored. Hence, their sensitivity to diverse antifungals can be determined using azoles, polyenes and echinocardins. In order to clarify the reason behind the poor biofilm and filamentation phenotypes of these mutants, their stress response under different osmotic and oxidative stress conditions can be explored in addition to the virulence in animal models *in vivo*. This knowledge will enable to design more effective antifungal therapies.

5.6.2 *Candida albicans – Pseudomonas aeruginosa* interaction studies *in vivo*

As the most infections are polymicrobial, the future studies should definitely focus on both physical and signal-mediated interaction between the microbes of the polymicrobial communities. The *in vivo* animal models including 2 pathogens are highly desirable because they can highlight more details of the infections (Lindsay and Hogan 2014). For example, our evidence suggests that *in vitro, P. aeruginosa* can significantly affect *C. albicans* and filamentous growth by its secreted molecules (Chapter 3). *In vivo* studies of their behaviour in animal models coinfected with both *C. albicans* and *P. aeruginosa* could further clarify the communication of these pathogens. To explore other elements involved in their communication the host needs to be included in the future experiments. The ultimate goal is to exploit this knowledge and devise virulence-limiting methods in order to benefit the infected patients.

5.6.3 *In vitro* validation of predicted *in silico* analyses

In silico studies of a master transcriptional regulator Efg1p, predicting its important role in yeast-hyphae switch as well as in *C. albicans – P. aeruginosa* interaction (Chapter 4), can be validated via *in vitro* analyses. To validate the *in silico* analysis of Efg1p across the *RBT* family gene promoter sequences (Chapter 4), *RBT* gene expression analysis can be conducted *in vitro*. Gene expression of *RBT1*, *RBT2*, *RBT4* and *RBT6* can be quantified in the *EFG1* mutant grown at 37^{o}C in YNBNP (filament-inducing medium) with RTqPCR including *EFG1* mutant grown at 30^{o}C in YNB (non-filament-inducing medium) as a control. The expression of the *RBT* genes is expected to be decreased in the *EFG1* mutant grown at 37^{o}C in YNBNP since Holcombe *et al*. (2010) have shown that both *RBT1* and *RBT4* were upregulated during filamentation of wild-type *C. albicans* and severely downregulated in the yeast conditions.

5.6.4 Updating and maintenance of the TFbsST database

A TFbsST database, developed here (Chapter 4), represents an important bioinformatics tool for *in silico* analysis of *C. albicans* promoter sequences and should be regularly updated in the framework of the future projects. To ensure operational efficiency, the TFbsST database should be updated regularly by populating the existing lists with more functionalities, TFs, experimentally tested motifs and *Candida*/non-*Candida* species. One of the functionalities that can be considered is an additional option for the user-defined motif that can be used to screen the promoter sequences. Constantly increasing lists of verified TFs and their motifs are good source of data and will need to be captured in the next versions of the TFbsST database. To accelerate *Candida* research, emerging pathogenic *Candida* species including *C. tropicalis* and *C. krusei*, need to be added to the TFbsST database list of organisms. An addition of an industrial yeast *Kluyveromyces marxianus* will make the TFbsST database useful bioinformatics tool for the remaining students in Dr. Morrissey's laboratory, who currently study different aspects of *K. marxianus*.

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