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Sexual Biofilm Formation in *Candida tropicalis* Opaque Cells

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Summary

Candida albicans and *Candida tropicalis* are opportunistic fungal pathogens that can transition between white and opaque phenotypic states. White and opaque cells differ both morphologically and in their responses to environmental signals. In *C. albicans*, opaque cells respond to sexual pheromones by undergoing conjugation, while white cells are induced by pheromones to form sexual biofilms. Here, we show that sexual biofilm formation also occurs in *C. tropicalis* but, unlike *C. albicans*, biofilms are formed exclusively by opaque cells. *C. tropicalis* biofilm formation was dependent on the pheromone receptors Ste2 and Ste3, confirming the role of pheromone signaling in sexual biofilm development. Structural analysis of *C. tropicalis* sexual biofilms revealed stratified communities consisting of a basal layer of yeast cells and an upper layer of filamentous cells, together with an extracellular matrix. Transcriptional profiling showed that genes involved in pheromone signaling and conjugation were upregulated in sexual biofilms. Furthermore, *FGR23*, which encodes an agglutinin-like protein, was found to enhance both mating and sexual biofilm formation. Together, these studies reveal that *C. tropicalis* opaque cells form sexual biofilms with a complex architecture, and suggest a conserved role for sexual agglutinins in mediating mating, cell cohesion and biofilm formation.

Introduction

Candida species are the fourth most common cause of bloodstream infections in hospital patients (Wisplinghoff *et al.*, 2004). While *Candida albicans* accounts for the majority of such infections, *C. tropicalis* is also commonly encountered in the clinic, particularly in individuals with hematologic malignancies (Sipsas *et al.*, 2009; Papon *et al.*, 2013). Multiple factors contribute to the ability of *Candida* species to infect the human host, including the capacity to form biofilms (Hasan *et al.*, 2009). Biofilms are complex structures involving both cell-cell and cell-substrate interactions. *C. albicans* biofilm organization typically consists of a basal layer of yeast cells upon which a mesh-like layer of hyphal and pseudohyphal cells develops together with an extracellular matrix (Chandra *et al.*, 2001; Bonhomme and d'Enfert, 2013). Biofilms are a growing concern in the medical community; they can be difficult to remove, can impede antifungal drug penetration, and can seed subsequent systemic infections (Donlan and Costerton, 2002; Blankenship and Mitchell, 2006; Finkel and Mitchell, 2011).

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Candida species have the ability to colonize multiple clinical devices, including venous and urinary catheters and prosthetics (Febré *et al.*, 1999; Crump and Collignon, 2000; Mohammadi *et al.*, 2013). Similarly, the majority of cases of denture-associated stomatitis, which affects approximately 65% of denture wearers, are caused by *Candida* species forming biofilms on these devices (Davenport, 1970; Gendreau and Loewy, 2011). *Candida* species have been observed to colonize other abiotic surfaces important for dental health such as stainless steel and porcelain (Ratnasari *et al.*, 2008; Li *et al.*, 2010). Thus, the factors allowing *Candida* species to form biofilms on synthetic surfaces are of direct relevance for preventing both mucosal and systemic infections by these pathogens.

C. albicans biofilm formation is strongly influenced by the phenotypic state of the cell. While *C. albicans* can undergo multiple forms of phenotypic switching, the best-characterized switch is the 'white-opaque' transition, which has also been observed in the related species *C. tropicalis* and *Candida dubliniensis* (Slutsky *et al.*, 1987; Pujol *et al.*, 2004; Porman *et al.*, 2011; Xie *et al.*, 2012). The white-opaque switch is an epigenetic, heritable transition, in which cells reversibly switch between the white state, where cells appear round and smooth, and the opaque state, where cells are elongated and pimpled (Slutsky *et al.*, 1987). In *C. albicans*, the two phenotypes are specialized for growth in different *in vivo* niches and respond differently to environmental stimuli. For example, while white cells are more adept at systemic infection, opaque cells are better suited for colonization of the skin (Kvaal *et al.*, 1997; Kvaal *et al.*, 1999; Xie *et al.*, 2013). In addition, white cells undergo filamentation in response to a variety of environmental cues, including high temperature and elevated CO_2 levels, while opaque cells undergo filamentation in response to distinct nutritional cues (Sudbery, 2011; Si *et al.*, 2013). Finally, the two cell types exhibit very different behaviors when challenged with sexual pheromones. While pheromones initiate a mating response in *C. albicans* opaque cells (Miller and Johnson, 2002), white cells respond to pheromones by becoming cohesive and adherent, forming a 'sexual' biofilm (Daniels *et al.*, 2006; Yi *et al.*, 2008; Lin *et al.*, 2013).

The regulation of sexual biofilm formation in *C. albicans* involves a pheromone-induced MAPK cascade (Daniels *et al.*, 2006; Yi *et al.*, 2008). Pheromones secreted by opaque cells interact with the G-protein coupled receptors Ste2 and Ste3 expressed on **a** and α cells, respectively. The pheromone signal is transduced via a heterotrimeric G protein complex and conserved MAPK cascade, culminating in activation of the transcription factor Cph1, an ortholog of *Saccharomyces cerevisiae* Ste12 (Bennett *et al.*, 2003; Yi *et al.*, 2008; Jones Jr. and Bennett, 2011; Lin *et al.*, 2013). *Candida* mating types are defined by transcription factors encoded at the mating-type-like (*MTL*) locus (Hull and Johnson, 1999; Butler *et al.*, 2009). Cells containing the *MTL***a** locus mate with cells containing the *MTL*α locus, while *MTL***a**/α cells are unable to mate (Hull *et al.*, 2000; Magee and Magee, 2000; Tsong *et al.*, 2003). Sexual biofilms in *C. albicans* are most efficient when formed by **a** or α white cells responding to pheromones secreted by opaque cells of the opposite mating type. These biofilms contrast with conventional (or asexual) biofilms that are pheromone-independent and formed preferentially by white **a**/α cells (Baillie and Douglas, 1999; Yi *et al.*, 2011; Bonhomme and d' Enfert, 2013). Studies comparing the genetic networks involved with sexual and asexual biofilm formation have found both shared and unique transcription

factors regulating the two biofilm models. For example, Cph1 and MAPK signaling are essential only for sexual biofilm formation, while a core set of transcription factors (including Bcr1, Brg1, Rob1 and Tec1) are required for both sexual and asexual biofilm formation (Sahni *et al.*, 2010; Nobile *et al.*, 2012; Lin *et al.*, 2013).

Why has the white-opaque switch evolved to regulate mating in a subset of *Candida* species? One proposal is that sexual biofilms formed by white cells are used to promote mating between rare opaque cells (Soll, 2009). Consistent with this model, experiments have established that sexual biofilms provide an optimal environment for mating to occur. Pheromone gradients accumulate to high concentrations within the sexual biofilm and aid chemotropic growth between opaque cells of opposite mating types (Daniels *et al.*, 2006; Park *et al.*, 2013). Thus, the *raison d'etre* for the white-opaque switch could be that sexual biofilms formed by white cells provide an appropriate environment for rare opaque cells to undergo successful conjugation *in vivo*.

Recently, a white-opaque phenotypic switch was discovered in *C. tropicalis* that shows similarities to that in *C. albicans* (Porman *et al.*, 2011; Xie *et al.*, 2012; Porman *et al.*, 2013). The master regulator of the white-opaque switch in both *Candida* species is the transcription factor Wor1 (Huang *et al.*, 2006; Zordan *et al.*, 2006; Srikantha *et al.*, 2006; Porman *et al.*, 2011; Xie *et al.*, 2012). In *C. albicans*, Wor1 acts as part of a transcriptional network to promote formation of the opaque state (Zordan *et al.*, 2007; Hernday *et al.*, 2013). In *C. tropicalis*, overexpression of *WOR1* was shown to drive switching to the opaque state as well as increase filamentation and biofilm formation (Porman *et al.*, 2013). These results indicate that significant crosstalk exists between the programs regulating the white-opaque switch and biofilm formation.

In this work, we address if mating pheromones can induce sexual biofilm formation in *C. tropicalis*. Similar to the case in *C. albicans*, we demonstrate that pheromone signaling in *C. tropicalis* drives the formation of sexual biofilms on synthetic surfaces. Surprisingly, however, *C. tropicalis* sexual biofilms are formed exclusively by opaque cells, and pheromone signaling is necessary but not sufficient for biofilm formation. This is in marked contrast to *C. albicans*, where activation of pheromone signaling in white cells was sufficient for induction of sexual biofilm formation. We also show that *C. tropicalis* sexual biofilms exhibit a stratified structure composed of a base layer of yeast-like cells, while the upper stratum is composed of highly filamentous cells. This structure contrasts with asexual biofilms induced by *WOR1* overexpression in *C. tropicalis*, which have a homogeneous makeup of filamentous cells surrounded by a more extensive extracellular matrix. Furthermore, our studies led to the identification of Fgr23, an ortholog of *S. cerevisiae* **a**-agglutinin, as a mediator of sexual mating and biofilm formation in *C. tropicalis*. These results are discussed with respect to the role of the white-opaque switch and agglutinin-like proteins in regulating biofilm formation in *Candida* species.

Results

C. tropicalis Opaque Cells Form Sexual Biofilms

White-opaque phenotypic switching was recently discovered in *C. tropicalis*, where cells in the opaque phenotypic state were shown to mate a hundred times more efficiently than cells in the white state (Porman *et al.*, 2011; Xie *et al.*, 2012). To determine if *C. tropicalis* exhibits the same capacity as *C. albicans* to form sexual biofilms, mixtures of **a** and α cells from both phenotypic states were tested in a modified biofilm assay. *C. tropicalis* cells were incubated in Lee's + Glucose medium on a polystyrene surface at 25°C for 48 hours, then non-adherent cells were removed by washing and the remaining adherent cells quantified. Mixtures of opaque **a** and α cells formed a robust biofilm under these conditions, while mixtures of white **a** and α cells did not (Fig. 1A). Analysis of the opaque biofilms revealed the presence of highly filamentous cells (49±1%). In contrast, cells of a single mating type produced very few filamentous cells on the polystyrene surface (Fig. 2D, 4±1%, p = 9.3E-6, Student's T-test), and no filamentous cells were produced in mixtures of white **a** and α cells (Fig. 1A).

In *C. albicans*, opaque cells secrete pheromones that can stimulate biofilm formation in responding populations of white cells. In particular, the presence of 2–10% opaque cells mixed with 90–98% white cells was reported to result in optimal sexual biofilm formation (Daniels *et al.*, 2006; Yi *et al.*, 2008). To test if a similar phenomenon occurs in *C. tropicalis*, white and opaque cells were coincubated at different ratios and the resulting biofilm formation was quantified. As shown by cell density measurements (Fig. 1B) and crystal violet staining (Fig. 1C), *C. tropicalis* biofilms did not form from mixtures of white **a** and α cells, whereas robust biofilms formed from mixtures of opaque **a** and α cells. Overall, the efficiency of biofilm formation increased as white cells were substituted with opaque cells in these assays (Fig. 1B and C). Similar sexual biofilms were formed when opaque cultures were grown at 30 or 37°C (Supp. Fig. 1). The latter result presumably reflects the stability of *C. tropicalis* opaque cells at 37°C (Porman *et al.*, 2011), whereas *C. albicans* opaque cells are unstable at this temperature and rapidly revert to the white state (Rikkerink *et al.*, 1988; Lohse and Johnson, 2010).

Next, to determine the role of mating type in *C. tropicalis* biofilm formation, assays were performed using populations of opaque cells with varying proportions of the two mating types. Both cell adherence (Fig. 2A and C) and crystal violet staining (Fig. 2B) were maximal when both **a** and α cells were present, whereas biofilms did not form when pure populations of either **a** or α cells were used. The requirement for both mating types is seen in the efficiency of overall biofilm formation and also at a cell morphology level; experiments involving only a single mating type yielded cells with a predominantly yeast morphology, whereas filamentous cells were prevalent in experiments containing mixtures of both mating types (Fig. 2D).

Given these results, we reasoned that pheromone signaling between opaque **a** and α cells could be important for *C. tropicalis* sexual biofilm formation. Pheromone signaling was previously shown to be essential for the formation of sexual biofilms in *C. albicans* (Daniels *et al.*, 2006; Yi *et al.*, 2008). We therefore tested *C. tropicalis* strains containing deletions in

the pheromone receptor genes *STE2* and *STE3* in **a** and α cells, respectively. *STE2* encodes the α pheromone receptor expressed on **a** cells, while *STE3* encodes the **a** pheromone receptor expressed on α cells, and loss of either receptor is sufficient to block mating (Porman *et al.*, 2011). Deletion of either *STE2* in **a** cells or *STE3* in α cells effectively abolished sexual biofilm formation in *C. tropicalis* (Fig. 3A). This result establishes that pheromone signaling is essential for sexual biofilm formation in *C. tropicalis*.

We next tested whether the addition of synthetic *C. tropicalis* pheromone was sufficient to drive biofilm formation in responding opaque cells. Several synthetic versions of *C. tropicalis* α pheromone were utilized including 13-mer, 15-mer and 16-mer (A-KF-KFRLTRYGWFSPN) peptides. Each of these peptides activated the *C. tropicalis* Ste2 receptor when heterologously expressed in *C. albicans* (Lin *et al.*, 2011). Synthetic pheromones were added to opaque **a** cells at concentrations up to 100 μg/ml but did not induce efficient biofilm formation (Fig. 3B). This is despite the fact that *C. tropicalis* **a** cells effectively formed mating projections in response to synthetic α pheromones (Supp. Fig. 2). We also tested whether biofilm formation was more efficient when cells were responding to natural pheromones secreted by both **a** and α cells. For this purpose, a transwell system was employed in which a semipermeable membrane separated a mixture of opaque **a** and α 'source' cells from a population of opaque **a** 'sink' cells contacting the polystyrene surface (Fig. 3D). Using this system, biofilms were not formed by 'sink' cells, although the induction of mating projections was not as strong as in response to synthetic pheromones or to co-cultures of **a** and α cells (Fig. 3C and data not shown). Together, these results suggest that pheromone signaling is necessary, but not sufficient, to induce efficient biofilm formation in *C. tropicalis* opaque cells.

Our results establish that *C. tropicalis* forms sexual biofilms between opaque **a** and α cells in a pheromone-dependent manner. However, direct cell-to-cell contact between **a** and α cells also appears to be necessary for efficient biofilm production. In contrast, pheromone alone is sufficient to stimulate cohesion and biofilm formation amongst responding white cells in *C. albicans* (Daniels *et al.*, 2006; Lin *et al.*, 2013). It therefore appears that the mechanisms regulating sexual biofilm formation exhibit significant differences between *C. tropicalis* and *C. albicans*.

C. tropicalis Sexual Biofilms Exhibit a Complex Structure

In *C. albicans*, the hallmarks of a mature biofilm include yeast and hyphal cell layers, as well as the presence of an extracellular matrix (Chandra *et al.*, 2001; Andes *et al.*, 2004; Finkel and Mitchell, 2011). To analyze the structure of *C. tropicalis* biofilms, scanning electron microscopy (SEM) and confocal laser scanning microscopy (CLSM) were employed. *C. tropicalis* sexual biofilms examined after co-culture of opaque **a** and α cells showed a bilayer structure; the cells closest to the polystyrene surface consisted mainly of chains of yeast cells and pseudohyphae, while the upper stratum consisted of much longer, filamentous cells (Fig. 4A, Supp. Fig. 3). Given that sexual biofilms are dependent on pheromone signaling, filamentous cells could include conjugation tubes, true hyphae, or pseudohyphae. Many of the long filaments contained septa with no obvious constrictions between cells, consistent with being true hyphae. However, other elongated cells did not

exhibit septation and showed morphologies more consistent with mating projections (Fig. 2D and 4A). We therefore suggest that a combination of filamentous forms contributes to biofilm structure.

To examine the frequency of mating within the sexual biofilm, auxotrophic opaque **a** and α strains were mixed and grown as a biofilm for 48 hours. Mating frequencies were determined by recovering cells and plating onto selective media (see Experimental Procedures). We observed that productive mating occurred within the sexual biofilm, with a mating frequency of 3.1% (±3.0%). While this indicates relatively efficient mating, a mating frequency of 23.2% (±11.5%) was observed when cells were co-incubated on Lee's + glucose agar plates under non-biofilm conditions (p= 0.031, Student's T-test). Thus, under the conditions tested, mating within a biofilm was less efficient than that between opaque cells mixed under standard *in vitro* conditions.

In order to gain a better understanding for how the sexual biofilm structure develops, SEM was performed on biofilms at different stages of development. At early time points (4 h), a layer of yeast cells was deposited onto the polystyrene surface (Fig. 5A, Supp. Fig. 4A). Adhesion occurred in a subset of cells, as the majority of the cells were washed away post-inoculation. The initial cell distribution was sparse, but by 12 hours of growth the base layer had spread to occupy the majority of the polystyrene surface. In several regions, filamentous cells had also begun to appear. Filamentous growth was extensive by 24 hours, by which time a meshwork of filamentous cells and extracellular matrix (ECM) had grown over the base layer of yeast cells, and by 48 hours the biofilm was completely covered by filamentous cells. Distinct cell morphologies were observed between the two layers of the biofilm; the upper layer was composed mainly of filamentous cells, while the lower layer was composed mainly of yeast cells (Fig 4A and 5A). Biofilm thickness ranged from 30 μm to 100 μm. In addition to extensive filamentous growth, the upper stratum contained a considerable amount of ECM material, as evidenced by clusters of fibrous material throughout this layer (Fig. 5A, Supp. Fig. 4A).

To further determine the distribution pattern of the ECM, cells were stained with the lectin Concanavalin A (ConA). ConA binds mannan and glucan polysaccharides that compose the majority of the cell wall and extracellular matrix (Mandal *et al.*, 1994; Chandra *et al.*, 2001; Gow and Hube, 2012). It appeared that ECM was primarily associated with filamentous cells of the outer layer, although matrix was also evident at appreciable levels throughout the biofilm (Fig. 4C). Matrix production was variable, with some filamentous cells brightly stained for ECM while others were not. An alternative possibility was that differential staining of filamentous cells was due to variable stain penetration, as previously observed in *C. albicans* (Yi *et al.*, 2011). To address this concern, confocal microscopy was performed on biofilms that were dispersed prior to fixation and staining (Supp. Fig. 5). ConA staining again localized to distinct subsets of cells, suggesting that some cells produce high quantities of ECM material or that ECM is preferentially bound to these cells.

Confocal microscopy was also performed on pure populations of opaque **a** cells that do not form a sexual biofilm. Although adhesion of some cells to the substrate was seen (Supp. Fig. 5D), dense growth did not occur, and there was a lack of ECM material. Furthermore,

filamentous growth was rare in these single-sex experiments, despite the fact that the distribution of adherent cells was similar to that of early biofilms containing both mating types. We conclude that ECM production and filamentous growth occurs during maturation of sexual biofilms, which is dependent on pheromone signaling between the two mating types.

WOR1 Overexpression Biofilms Exhibit Distinct Structures from Sexual Biofilms

In *C. tropicalis*, overexpression of *WOR1*, the master transcriptional regulator of the white-opaque switch, was recently shown to induce filamentation on solid media and biofilm formation in cells grown on a polystyrene substrate (Porman *et al.*, 2013). These biofilms involved opaque cells expressing *WOR1* under the control of the strong constitutive promoter, *TDH3*, and were characterized by extensive filamentation, demonstrating links between the white-opaque switch, filamentation, and biofilm formation. In contrast to sexual biofilms, however, *WOR1* overexpression biofilms were formed in cultures of a single mating type (opaque **a**, α, or **a**/α cells) (Porman *et al*., 2013). To determine if these biofilms were dependent on pheromone signaling, *WOR1* was also overexpressed in cells lacking the Ste2 receptor. Despite the absence of the pheromone receptor, these cells produced biofilms that were indistinguishable from wildtype cells overexpressing *WOR1* (data not shown). Thus, in contrast to the sexual biofilms formed by mixed cultures of opaque **a** and α cells, the biofilms formed by *WOR1* overexpression are asexual biofilms.

We directly compared the structures of sexual biofilms with *WOR1* overexpression biofilms using SEM and CLSM. Several notable differences were evident between the two models. First, whereas in sexual biofilms filamentous growth was restricted to the upper stratum of the biofilm, in *WOR1* overexpression biofilms filamentation occurred throughout the biofilm structure (compare Fig. 4A and B, and Fig. 4C and D). Second, extensive filamentous growth occurred at earlier time points in the *WOR1* overexpression biofilm (see 12 h time points in Fig. 5 and Supp. Fig. 4), consistent with high *WOR1* expression enhancing filamentation (Porman *et al*., 2013). Third, ECM was consistently distributed in the *WOR1* overexpression biofilms, whereas ECM production was more variable in the sexual biofilms (Fig. 4A and B). Quantification of ECM content by a Glucatell assay confirmed that both biofilm types contained more ECM than wildtype opaque **a** cells alone ($p<0.01$, Tukey's test), and that *WOR1* overexpression biofilms contained 36% more ECM than sexual biofilms, although this difference was not significant (Supp. Fig. 6). Despite these structural differences, overall biofilm thicknesses were similar in the two models as determined by SEM (Fig. 4). Together, these results demonstrate that *C. tropicalis* can form biofilms with different structural features, as exemplified by sexual and *WOR1* overexpression biofilms.

Transcriptional Profiling of C. tropicalis Biofilm Formation

Cells within biofilms often exhibit distinct properties compared to cells grown under planktonic conditions. Transcriptional profiling was therefore used to compare non-biofilm populations (white *wor1*Δ **a** cells or opaque **a** cells), sexual biofilm populations (mixtures of opaque **a** and α cells), and *WOR1* overexpression biofilms (opaque **a** cells overexpressing the *WOR1* transcription factor). RNA was recovered from cells grown under each of these

conditions, and the resulting cDNA was hybridized to custom *C. tropicalis* microarrays (see Experimental Procedures).

Most informative were the profiles of sexual and *WOR1* overexpression biofilms compared to that of planktonic opaque **a** cells that do not form biofilms under the assay conditions (Fig. 6A). In this comparison, sexual biofilms upregulated 68 genes and downregulated 11 genes (>2 fold change), while *WOR1* overexpression biofilms upregulated 21 genes and downregulated 37 genes (Fig. 6C, Supp. Table 3). Significant overlap was found between sets of biofilm-regulated genes with five genes exhibiting similar expression changes in the two biofilm populations (p= 6.9E-4, hypergeometric distribution, Supp. Table 4). Shared upregulated genes included *EFH1*, whose ortholog regulates filamentation in *C. albicans*, and *CTRG_04837*, whose ortholog (orf19.2457) is upregulated in conventional *C. albicans* biofilms (Doedt *et al.*, 2004; Nobile *et al.*, 2012). In addition, *CTRG_03464* was downregulated in both *C. tropicalis* biofilm types, although its *C. albicans* ortholog (orf19.5282) is upregulated in rat biofilm models in *C. albicans* (Nett *et al.*, 2009).

We also directly compared the transcriptional profile of *C. tropicalis* sexual biofilms to those formed by *C. albicans* (Supp. Table 5). We found that 13% of the genes upregulated in *C. tropicalis* sexual biofilms were also induced in *C. albicans* sexual biofilms (Lin *et al.*, 2013), a significant overlap (p=3.3E-5, hypergeometric distribution, Supp. Table 4 and 5). This is in spite of the fact that sexual biofilm formation in *C. albicans* occurred in white cells whereas *C. tropicalis* sexual biofilms occurred in opaque cells. In contrast, a comparison of upregulated genes between *C. tropicalis* sexual biofilms and conventional *C. albicans* biofilms (Nobile *et al.*, 2012) revealed no significant overlap (p=0.17, Supp. Table 4 and 5).

GO-Term analysis was performed on *C. tropicalis* sexual biofilm genes when compared either to the white control strain (Supp. Table 6) or the opaque control strain (Supp. Table 7). In these analyses, significant gene expression changes included those associated with multi-organism processes, pheromone response, conjugation, and biofilm formation. These results indicate that *C. tropicalis* biofilms undergo significant transcriptional changes in genes involved in specialized growth and communication in order to establish the new community structure. Sexual *C. tropicalis* biofilms showed specific induction of many genes associated with mating, including FIG1, FUS1, and *KAR4*. These sex-related genes accounted for 20 of the 55 genes with known orthologs that were upregulated greater than 2-fold in the sexual biofilm assays (Supp. Table 7), and establish a strong connection between the mating response and sexual biofilm formation in *C. tropicalis* (Fig. 6B).

WOR1 overexpression biofilms also included gene expression changes that were not observed in sexual biofilms. For example, *WOR3*, *CHT3*, *JEN2*, *CTRG_05754*, and *CTRG_05849* were all regulated only in the *WOR1*-mediated biofilms (Fig. 6B). The transcriptional regulator *WOR3* is known to be highly upregulated in both *C. albicans* opaque cells compared to white cells and in cells grown within biofilms (Nobile *et al.*, 2012; Lohse *et al.*, 2013). This was not the case in *C. tropicalis*, however, since planktonic opaque cells showed decreased expression of *WOR3* relative to white cells (Porman *et al.*, 2013),

and *WOR3* expression was further decreased in *WOR1*-overexpressing biofilms compared to control opaque cells (Fig. 6B).

Analysis of the Role of FGR23 and EFH1 in C. tropicalis Biofilm Formation

Cell adhesion and cell fusion are critical steps in the mating process, and it is possible that the physical interactions that facilitate these processes promote biofilm formation. We noted that *C. tropicalis FGR23* (*CTRG_02409*) is strongly induced in sexual biofilms (5.5-fold) as well as weakly induced in *WOR1* overexpression biofilms (1.4-fold). The *FGR23* gene is an ortholog of *S. cerevisiae AGA1*, which encodes the anchorage subunit for the **a**-agglutinin heterodimer (Roy *et al.*, 1991). *AGA1* is expressed in both **a** and α cell types of *S. cerevisiae* and is also highly induced in response to pheromone (Roberts *et al.*, 2000; Guo *et al.*, 2000; Dranginis *et al.*, 2007). Aga1 is a glycophosphatidylinositol-linked protein that anchors Aga2 to the cell surface of **a** cells, where it interacts with the complementary α-agglutinin expressed on the surface of α cells. Contacts between agglutinins facilitate the interaction of *S. cerevisiae* mating partners, and thus loss of Aga1 results in a reduction of mating efficiency in liquid medium (Roy *et al.*, 1991; de Nobel *et al.*, 1995). Deletion of Aga1 also disrupts mating on solid medium if combined with deletion of the related adhesin, Fig2 (Guo *et al.*, 2000).

To determine if Fgr23 facilitates mating or biofilm formation in *C. tropicalis*, we generated *fgr23*Δ and *FGR23* over-expression (*pTDH3-FGR23*) strains. Deletion of *FGR23* resulted in a decrease in sexual biofilm production, as co-culture of **a** and α *fgr23*Δ strains generated biofilms that were significantly reduced compared to those formed by wild type strains (Fig. 7, p= 1.2E-4, Student's T-test). Interestingly, deletion of *FGR23* from either **a** or α cells alone also resulted in a defect in biofilm formation (data not shown). Reintegration of *pTDH3-FGR23* into the deletion strain background significantly increased biofilm formation by 43% compared to deletion strains (p=0.049, Student's T-test), partially restoring the wildtype phenotype. In contrast, integration of the *pTDH3-FGR23* construct into the wildtype background did not significantly alter biofilm formation compared to wildtype (data not shown).

The role of *FGR23* in mediating mating in *C. tropicalis* was also addressed. Wildtype cells and *fgr23*Δ mutants were compared for mating efficiency when **a** and α cells were co-incubated on Spider medium. Mutants lacking *FGR23* were still able to fuse and mate, although mating efficiency was reduced by an order of magnitude between *fgr23*Δ cells under standard conditions (Supp. Fig. 7). This indicates that Fgr23 plays an important role in promoting both biofilm formation and conjugation in *C. tropicalis*.

Filamentation is an integral part of biofilm formation in *Candida* species, and many mutants defective in filamentation are unable to form biofilms or mount successful infections (Lo *et al.*, 1997; Ramage *et al.*, 2002; Nobile *et al.*, 2006; Laforet *et al.*, 2011). In *C. albicans*, *EFH1* is a regulator of filamentous growth and works in concert with the related APSES transcription factor *EFG1* (Doedt *et al.*, 2004). Given that *EFH1* was upregulated approximately 2-fold in both sexual and *WOR1*-overexpression biofilms, the *EFH1* gene was deleted to determine its role in biofilm formation. However, deletion of *EFH1* had no

significant effect on sexual biofilm formation (Supp. Fig. 8). This could be due to functional redundancy with a yet-to-be identified transcription factor, or could simply indicate that *EFH1* does not contribute to biofilm efficiency.

Discussion

Here, we report that *C. tropicalis* forms a sexual biofilm as a result of pheromone signaling between **a** and α cells. Biofilm formation required that cells be in the opaque phenotypic state, and cells in the white state did not contribute to overall biofilm formation. Structural analysis of the biofilms revealed them to be highly stratified, with the lower layer of the biofilm consisting mostly of yeast cells, while the upper stratum contained primarily filamentous cells. The latter included a mixture of both hyphae and conjugation tubes, and extracellular matrix was associated with sub-populations of filamentous cells. Overall, the structure of *C. tropicalis* sexual biofilms showed physical similarities to both sexual and asexual biofilms formed by *C. albicans*. These, too, consist of a basal layer of yeast cells upon which a meshwork of filamentous cells develops together with associated ECM material (Chandra *et al.*, 2001; Daniels *et al.*, 2006; Bonhomme and d' Enfert, 2013; Daniels *et al.*, 2013).

Previously, it was shown that *C. albicans* also forms a sexual biofilm (Daniels *et al.*, 2006). In this species, biofilms result from white **a** or α cells responding to sexual pheromones produced by opaque cells of the opposite sex (Daniels *et al.*, 2006; Park *et al.*, 2013). Thus, the most efficient biofilms are generated by cultures containing a majority of white **a** and α cells mixed with a minority of opaque cells as a source of pheromone (Daniels *et al.*, 2006). In both white and opaque cells, pheromone signaling occurs via a conserved MAPK cascade that culminates in activation of the Cph1/Ste12 transcription factor (Yi *et al.*, 2008; Sahni *et al.*, 2010; Lin *et al.*, 2013). Additional factors regulating *C. albicans* sexual biofilm formation include the transcription factors Bcr1, Brg1, Rob1 and Tec1, downstream effectors such as the G1 cyclin-related protein Hgc1, as well as cell surface proteins Eap1, Csh1, and Pga10 (Sahni *et al.*, 2009; Sahni *et al.*, 2010; Lin *et al.*, 2013).

The current studies establish that while both *C. albicans* and *C. tropicalis* form sexual biofilms, the regulation of biofilm formation has diverged since these species last shared a common ancestor. In *C. albicans*, pheromones induce white cells to produce robust biofilms, whereas opaque cells form weak, unstable biofilms (Daniels *et al.*, 2006; Lin *et al.*, 2013). The formation of sexual biofilms was shown to promote *C. albicans* mating *in vitro* (Park *et al.*, 2013), as biofilms stabilized pheromone gradients between opaque cells, thereby promoting chemotropism between potential mating partners (Daniels *et al.*, 2006). Given the low frequency of *C. albicans* mating in natural populations, it was suggested that the white-opaque switch has been retained in this species as a mechanism for promoting biofilm formation rather than for directing rare mating events (Soll, 2009).

In contrast to *C. albicans*, we demonstrate that sexual biofilms in *C. tropicalis* are formed exclusively by cells in the opaque state. In addition, whereas substrate adhesion in *C. albicans* cells can be induced by pheromone alone (Daniels *et al.*, 2006), biofilm formation in *C. tropicalis* was most efficient when there was physical contact between **a** and α cells.

Taken together, our studies establish that opaque cells of *C. tropicalis* are both the mating-competent form *and* the form most capable of generating a sexual biofilm. Furthermore, the thermostability of the opaque state in *C. tropicalis* allows sexual biofilms to form equally well at both 25°C and 37°C, potentially making sexual biofilm formation relevant for infections in the clinic.

Sexual biofilms in *C. tropicalis* were also compared to asexual biofilms formed due to overexpression of Wor1, the master regulator of the white-opaque switch. Wor1 forms part of a network of interacting transcriptional feedback loops that promotes stable and heritable formation of the opaque state (Huang *et al.*, 2006; Zordan *et al.*, 2006; Srikantha *et al.*, 2006; Zordan *et al.*, 2007; Hernday *et al.*, 2013). While *C. tropicalis* white cells and single-sex cultures of opaque cells formed insubstantial biofilms, overexpression of Wor1 in **a**, α, or **a**/α cell types induced filamentation and resulted in biofilms that were comparable in size to sexual biofilms. Interestingly, the structures of Wor1-mediated and sexual biofilms were distinct. Whereas sexual biofilms showed clear stratification and variable ECM density, Wor1-mediated biofilms exhibited more homogeneous filamentation throughout the biofilm structure.

Expression profiling of *C. tropicalis* cells grown in the two biofilm models revealed both similarities and significant differences between these models. For example, only sexual biofilms were associated with the induction of mating genes, including pheromone and pheromone receptor genes, consistent with the key role of mating signaling in generating these biofilms. *WOR1* overexpression and sexual biofilms also upregulated a shared set of genes, which included *FGR23, EFH1, XOG1* and several uncharacterized genes. *XOG1* encodes a glucanase that may potentially affect adhesion of cells to the substrate surface (González *et al.*, 1997), although this possibility was not tested here.

The potential roles of *FGR23* and *EFH1* in sexual biofilm formation were further assessed by genetic analysis of these two genes. *EFH1* is a transcription factor regulating filamentous growth in *C. albicans* (Doedt *et al.*, 2004), yet deletion of this gene in *C. tropicalis* did not lead to an obvious colony or cellular phenotype and did not alter sexual biofilm formation. In contrast, deletion of the *C. tropicalis FGR23* gene resulted in a significant decrease in sexual biofilm formation. *FGR23* is an ortholog of *AGA1* in *S. cerevisiae*, which encodes a component of the mating-type **a**-agglutinin. *S. cerevisiae* agglutinins consist of **a**-agglutinin, formed by a complex of Aga1 and Aga2, and the α-agglutinin, Sag1. These cell surface factors bind one another irreversibly during mating to promote conjugation (Lipke and Kurjan, 1992; Cappellaro *et al.*, 1994; Dranginis *et al.*, 2007). Significantly, the *C. albicans* Agglutinin-Like Sequence (ALS) gene family shows homology to *S. cerevisiae* α-agglutinin and this family plays a prominent role in *C. albicans* biofilm formation (García-Sánchez *et al.*, 2004; Silverman *et al.*, 2010).

We demonstrate that the **a**-agglutinin-related protein, Fgr23, is necessary for efficient formation of sexual biofilms by *C. tropicalis* opaque cells. *C. tropicalis fgr23*Δ mutants also showed a reduced mating efficiency compared to wildtype cells, indicating that the function of *FGR23* may be conserved with that of *S. cerevisiae AGA1*. In *S. cerevisiae*, *aga1* mutants show mating defects that are highly exacerbated by additional loss of the related FIG2 gene

(Guo *et al.*, 2000). In fact, *S. cerevisiae* proteins with adhesive properties are often interchangeable, even if they exhibit considerable differences at the amino acid sequence level. Thus, adhesins that function in flocculation can substitute for adhesins that promote mating and vice versa, if expressed at a high enough level and localized appropriately (Guo *et al.*, 2000). It is therefore apparent that adhesins can play diverse roles in mating, flocculation, and filamentation, and that these functions depend more on their expression pattern than their primary sequence. In the case of *C. tropicalis*, the function of Fgr23 further supports these conclusions, as this protein promotes both mating and sexual biofilm formation. Additional experiments will be required to address the specific role of Fgr23, and whether this factor acts to promote cell-cell or cell-substrate interactions to enhance biofilm formation by this pathogen.

Experimental Procedures

Media

Preparation of YPD and SCD followed established protocols (Guthrie and Fink, 1991; Liu *et al.*, 1994). Nourseothricin resistant strains (SAT^R) were selected on yeast extract peptone dextrose (YPD) plates containing 200 μg/ml nourseothricin. Spider medium contained 1% nutrient broth (v/v), 0.4% potassium phosphate (w/v), 2% mannitol (pH 7.2) (w/v), and 1.35% agar (w/v). Lee's medium was formulated as previously described (Lee *et al.*, 1975) with leucine, arginine and histidine added at 0.13%, 0.01% and 0.01%, respectively.

Strain and Plasmid Construction

Strains used in the current experiments are listed in Supplemental Table 1. *WOR1* over-expression and deletion strains were constructed as described (Porman *et al.*, 2013). Opaque strains were obtained by plating white strains on Lee's + N-acetyl glucosamine medium for 2–7 days, then re-plating on Spider medium for 2–7 days and selecting opaque colonies following visual and microscopic examination of phenotype.

Deletion of *FGR23* and *EFH1* was performed using a previously described method (Noble and Johnson, 2005). 5′ and 3′ regions flanking the ORF (open reading frame) were PCR amplified (Supp. Table 2). *HIS1* and *ARG1* auxotrophic markers were PCR amplified from plasmids pSN64 (*ARG4*) and pSN52 (*HIS1*). ORF flanks were then combined with the auxotrophic markers using a fusion PCR protocol (Noble and Johnson, 2005). The resulting PCR product was used to transform auxotrophic strains. Correct integration was determined by PCR across the junctions of the integrating construct. The process was repeated to delete the second copy of the ORF, and then ORF PCR was performed to confirm deletion of the gene.

FGR23 complementation strains were constructed by PCR amplification of the *FGR23* ORF and the *TDH3* promoter, which were subsequently joined in a fusion PCR step. The PCR product was cloned into pSFS2a (Reuss *et al.*, 2004) using *Kpn*I and *Xho*I restriction sites. The resulting plasmid was used to transform wild type and *fgr23* deletion strains. Integration was confirmed by PCR.

Biofilm Adherence Assay and Biofilm Staining

Strains were grown overnight at 25°C in Spider medium. Cells were centrifuged and resuspended in Lee's + Glucose medium. One ml of Lee's + Glucose medium was added per well of a 12-well plate. Either a single mating type or combinations of each mating type were added to each well for a total of 4×10^7 cells per well. Plates were incubated at room temperature for two days unless otherwise stated. Each well was then gently washed thrice with 1 ml of Phosphate-Buffered Solution (PBS) to remove non-adherent cells. Biofilms were imaged using a Bio-Rad Chemi-Doc imager. Optical density of biofilms was determined by scraping off adherent cells and resuspending them in PBS, diluting appropriately, and measuring absorbance at 600 nm on a Thermo-Scientific Nanodrop 2000c spectrophotometer.

Crystal violet staining was performed by decanting the wells and drying the adherent cells for 45 minutes. Cells were stained for 45 minutes with 385 μl of 0.4% aqueous crystal violet per well, followed by three 1 ml washes with water and destaining with 700 μl of 95% ethanol. The destain solution was diluted 10-fold into a 96-well plate, and absorbance at 595 nm was read using a BioTek Synergy HT plate reader.

Transwell Assay

Strains were grown overnight at 25°C in Spider medium. Cells were then centrifuged and resuspended in Lee's + Glucose medium. One ml of Lee's + Glucose medium was added to the lower region of each well of a transwell plate (Costar Transwell, 0.4 μm polycarbonate membrane,12-well), along with 2×10^7 cells of a single mating type. Then, 0.5 ml of Lee's + glucose medium was added to the inner chamber, along with 2×10^7 cells of the opposite mating type, or a mix of the two mating types. Plates were incubated at room temperature for two days unless otherwise stated. The transwell chambers were removed, and the wells were washed and treated similarly to the previously described adherence assay.

Mating Assay

Auxotrophic strains were grown overnight at 25°C in Spider medium. Cultures were centrifuged and resuspended in either Lee's + glucose medium or Spider medium as indicated. Approximately $2*10^7$ cells of each mating type were spotted onto a filter (Millipore Cat. # AAWG01300) on a Lee's + glucose agar plate, a Spider agar plate (if Spider liquid was used), or cultured in the above-described sexual biofilm assay. After two days, cells were scraped from the filters or biofilms and resuspended in 1 ml of water. Cells were diluted appropriately and plated on selective media to select for parental strains or progeny. CFUs were counted after two days, and mating success was calculated by dividing the number of progeny by the limiting parent.

Zygote staining

Cultures were grown overnight at 25°C in Spider medium, then centrifuged and resuspended in water. FITC-Concanavalin A (**a** strains) or Rhodamine-Concanavalin A (α strains) was added to each culture at a concentration of 25 μg/ml, and cells stained for one hour at 25°C. Cells were washed and 10^7 of each mating type were combined and plated onto a filter on

Spider medium for one day at 25°C. Cells were recovered from the filters, resuspended in water, and analyzed via fluorescence microscopy using a Zeiss Axio Observer Z1.

Microscopy and Imaging

For scanning electron microscopy, biofilms were prepared similarly to the adherence assay except a 1 cm^2 polystyrene square cut from a tissue-culture lid was added to each well prior to the addition of cells so that the majority of the biofilm formed on the square. Each biofilm square was fixed with 2.5% (w/v) glutaraldehyde in buffer (0.1 M Na-cacodylate, pH 7.4) overnight at 4°C. Squares were washed with the buffer, then subjected to 1% aqueous osmium tetroxide in buffer at 25°C for 90 minutes, then washed again. Squares were dehydrated using a 15% gradient ethanol series, dried in a critical point dryer, and finally coated with 20 nm 60:40 gold:palladium in an Emitech K550 sputter coater. Imaging was performed using a Hitachi S-2700 and Quartz PCI software.

For confocal microscopy, biofilms were also prepared on polystyrene squares, however they were fixed overnight in 4% formaldehyde at 4°C. Squares were then washed with water, followed by staining in 1 ml of 25 μg/ml Rhodamine-conjugated Concanavalin A for one hour in the dark at 25°C. Next, they were stained with 50 μg/ml Calcoflour White for one minute and washed with 1 ml water. Squares were then inverted onto coverslips and microscopy was performed on a Zeiss LSM 510 META microscope. Microscope settings were static across samples so that direct comparisons of staining intensities could be made when possible.

To image individual cells, biofilms were prepared similarly to the adherence assay, however, any staining was performed on the wells following washing, and then cells were scraped from the wells and suspended in approximately 10 μl water on a glass slide. Differential Interference Contrast (DIC) and fluorescence images were collected using a Zeiss Axio Observer Z1.

RNA Purification and Microarray Analysis

Biofilms were prepared similarly to the adherence assay, except after the two-day incubation period biofilms were not washed. Rather, adherent cells were resuspended in the medium with non-adherent cells. RNA was extracted from cells using the RiboPure-Yeast Kit (Ambion). DNA was removed using DNAseI (Ambion) followed by phenol/chloroform extraction. Aminoallyl-labeled cDNA was generated from the purified RNA, and then hybridized to a custom *C. tropicalis* Agilent microarray, as previously described (Porman *et al.*, 2011). The microarrays were read and quantified using a Genepix 4000 scanner with GENEPIX PRO v3.0 (Axon Instruments) software. Data was then normalized using Goulphar web-based software (http://transcriptome.ens.fr/goulphar) with the following conditions: Foreground – Median, Background – Median background subtraction, Normalization – Print-tip group lowess (200), Flags – filter all, Saturating spots – remove if intensity is >50k in a single channel. Data was organized, averaged (two replicates each) and labeled in Microsoft Excel using known *C. albicans* or *S. cerevisiae* gene ortholog names for unannotated genes (candidagenome.org, yeastgenome.org, cgob3.ucd.ie, or www.broadinstitute.org/regev/orthogroups). Data was analyzed using Cluster v2.11 and

visualized using TreeView v1.60 (http://rana.lbl.gov/EisenSoftware.htm). Cluster was also used for filtering results, where differentially regulated genes were selected by applying a >2-fold change cutoff. Array data is available from GEO (GSE52634 and GSE43267). Gene ontologies were determined using the CGD Gene Ontology Mapper (www.candidagenome.org/cgi-bin/GO/goTermMapper), and p-values for GO terms were calculated using a hypergeometric distribution with Multiple Hypothesis Correction. The p-value for comparing overlap between the sexual and *WOR1* overexpression biofilms was generated using a hypergeometric distribution in Microsoft Excel.

Extracellular Matrix Quantification

Extracellular matrix was quantified similarly to previously described methods (Taff *et al.*, 2012) using the Glucatell © assay (Associates of Cape Cod, Inc. Falmouth, MA). Briefly, biofilms formed in the biofilm adherence assay were scraped and resuspended in the supernatant. Next, samples were sonicated for 10 minutes to disassociate cells and matrix material, and then centrifuged three times at 4,500x*g* for 20 minutes to remove cells. Glucan concentration of the recovered supernatants was then determined per the manufacturer's supplied protocol.

Statistical Analysis

Statistical analysis was performed using the PAST software package (folk.uio.no/ohammer/past/). All datasets were tested for normality using the Anderson-Darling method, and then the appropriate tests were performed. Tests across sample sets, such as one-way ANOVA or Kruskal-Wallis, were performed first, followed by between-sample tests, including Student's T-test, Tukey's test or Mann-Whitney test, as indicated in figure legends.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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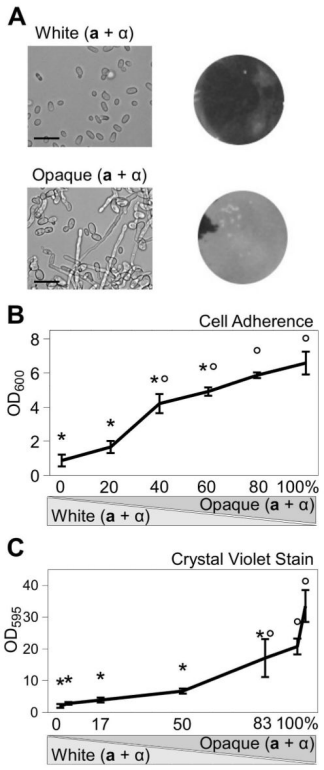

Figure 1.

C. tropicalis opaque cells form sexual biofilms. Mixtures of white and opaque **a** and α cells were compared for their ability to form biofilms. (A) A 50:50 mix of **a** and α cells were grown on polystyrene plates for 48 h and non-adherent cells removed by washing. Mixtures of white **a** and α cells showed little adherence to the plastic while a mixture of opaque **a** and α cells formed an adherent layer on the plastic. Left side, DIC microscopy of cells scraped from biofilm-inducing conditions, scale bar = 15 μm; right side, images of biofilms following washing of the plate. Black areas indicate unbound polystyrene while lighter-colored areas show adherent *Candida* cells. (B and C) White and opaque cell populations were mixed together at the indicated ratios. A 50:50 ratio of **a** and α cells was used for these experiments. Adherent cells were resuspended and quantified by absorbance at 600nm (B) or by crystal violet staining then absorbance at 595nm (C). Mean ± standard deviation (N≥4). * $p<0.005$ compared to 100% opaque cells. ° $p<0.005$ compared to 100% white cells (Tukey's test).

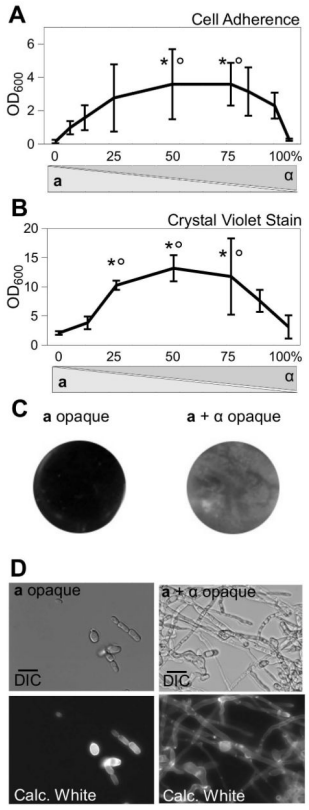

Figure 2.

Equal proportions of **a** and α cells yield maximum sexual biofilm formation. After growth of opaque cells for 48 h in polystyrene plates, the plates were washed to remove non-adherent cells then (A) adherent cells were resuspended and quantified by absorbance at 600nm, or (B) adherent cells were stained with crystal violet and quantified by absorbance at 595nm. Mean ± standard deviation (N≥3). * $p<0.05$ compared to 100% **a** cells. ° $p<0.05$ compared to 100% α cells. Tukey's test. (C) Images of biofilms following washing of the plate. Black areas indicate unbound polystyrene while lighter-colored areas show adherent *Candida* cells. (D) Microscopy of cells scraped from biofilm-inducing conditions. Top panels are DIC images and bottom panels are corresponding calcofluor white-stained cells. Scale bars = 15 μm.

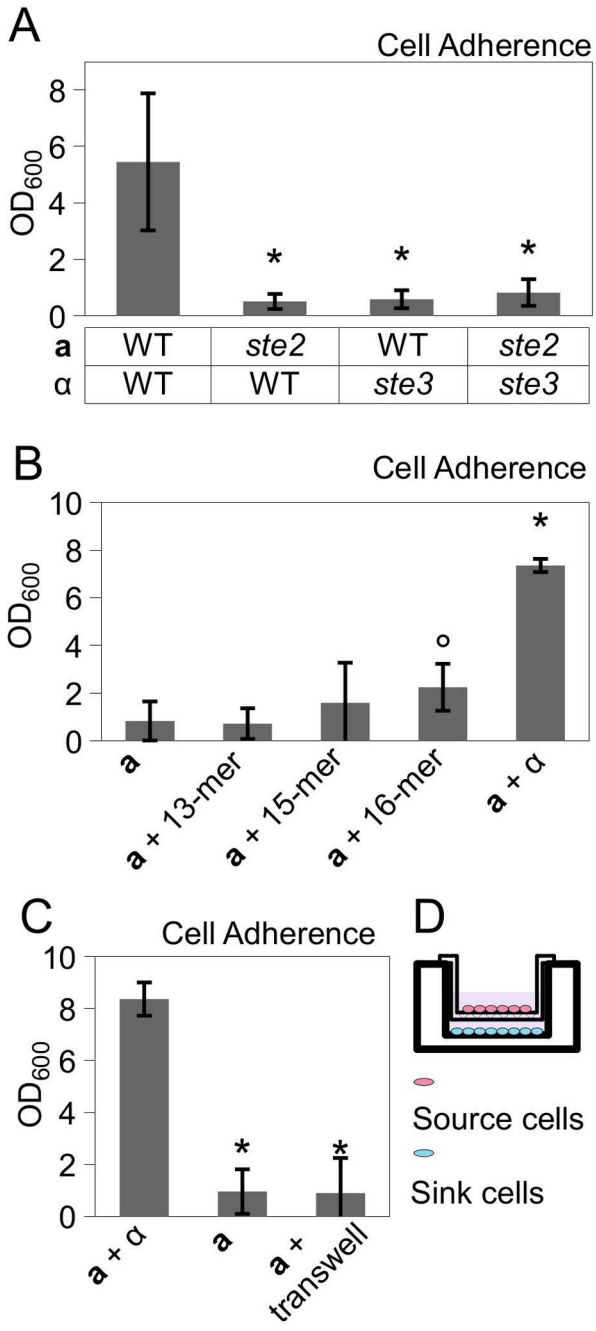

Figure 3.

Pheromone signaling is necessary, but not sufficient, for sexual biofilm formation by *C. tropicalis* opaque cells. (A) Quantification of biofilm formation by *C. tropicalis* strains lacking pheromone receptors for α pheromone (Ste2) or for **a** pheromone (Ste3). * $p<0.001$. Mann-Whitney test. (B) Biofilm formation by *C. tropicalis* **a** cells supplemented with synthetic α pheromone at 100 μg/ml (13-mer, 15-mer or 16-mer) or α cells. ° $p<0.05$ compared to **a** cells alone. * $p<0.01$ compared to **a** cells alone. Mann-Whitney test. (C) Biofilm formation of **a** cells (sink cells) exposed to pheromones produced by a mixed culture of opaque **a** and α cells (source cells), that were separated from sink cells by a semi-permeable transwell. * $p<0.001$, Tukey's test. (D) Schematic diagram of transwell setup. Biofilm measurements show mean ± standard deviation ($N\geq4$).

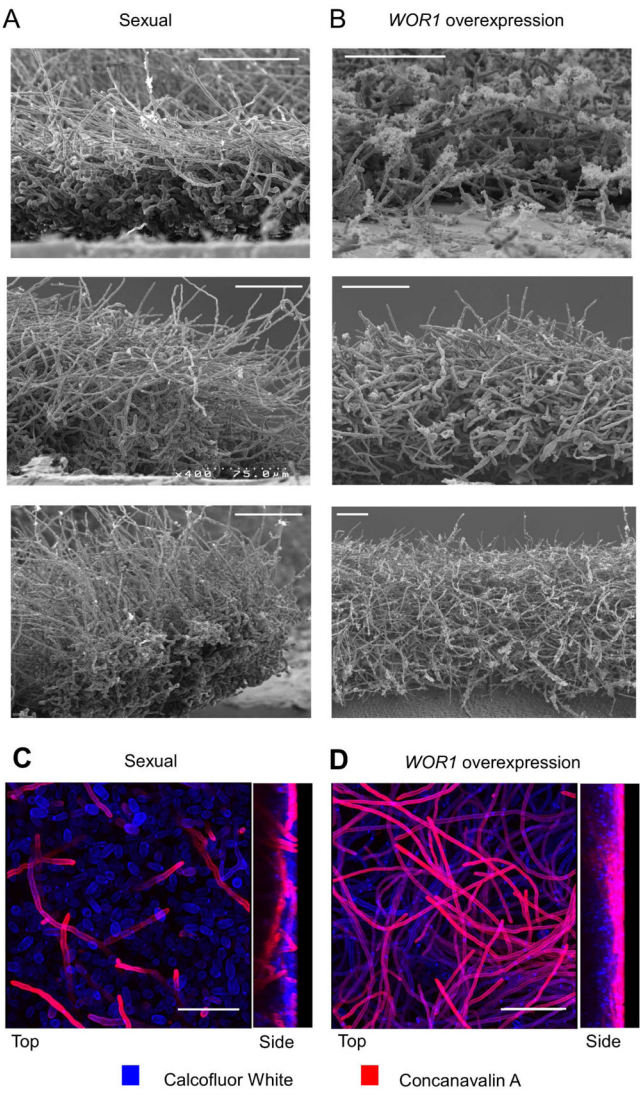

Figure 4.

Microscopic analysis of *C. tropicalis* sexual and *WOR1* overexpression biofilms. Scanning electron micrographs showing side views of biofilms formed after 48 hours of development. (A) *C. tropicalis* **a** and α opaque cells (sexual biofilm). (B) *WOR1* overexpression biofilm. 600X (top image), 400X (middle and bottom left images) or 200X magnification (bottom right). Scale bar = 60 μm (valid only in foreground). Confocal imaging of (C) sexual and (D) *WOR1* overexpression biofilms. Left, top views projected from outer biofilm surface inward. Right, side views of biofilms. Images were taken with the same settings to allow for direct comparison. Scale bar = 36 μm. Blue = Calcoflour White. Red = Rhodamine conjugated Concanavalin A.

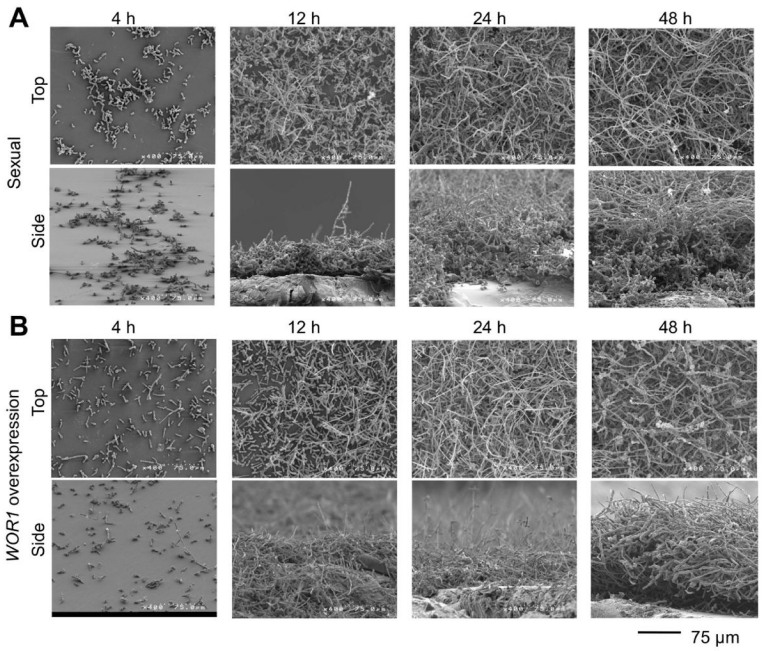

Figure 5.

Time course of *C. tropicalis* biofilm development. (A) Representative SEM images of sexual biofilms consisting of a 50:50 mixture of opaque **a** and α cells. (B) Representative SEM images of biofilms from opaque cells overexpressing *WOR1*. Cells were grown on a polystyrene substrate and then samples were analyzed by scanning electron microscopy at 4, 12, 24, and 48 h following inoculation. Magnification = 400X. Scale bars = 75 µm.

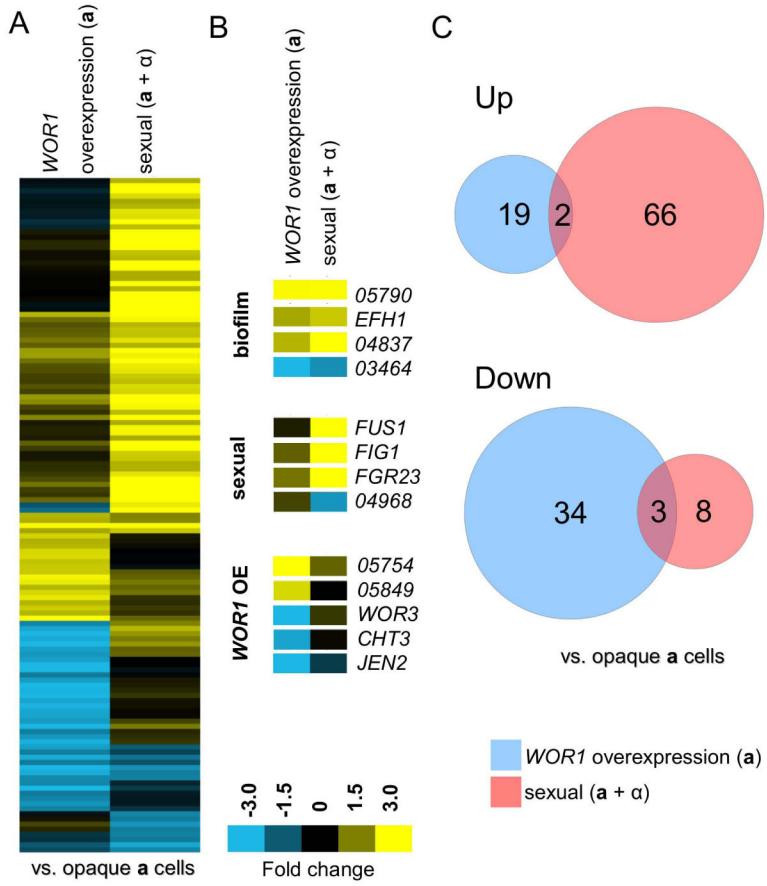

Figure 6.

Transcriptional profiling of *C. tropicalis* sexual and *WOR1* overexpression biofilms. (A) Gene expression profiles comparing a *WOR1* overexpression biofilm (p*TDH3*-*WOR1* in opaque **a** cells) and a sexual biofilm (consisting of a 50:50 mixture of opaque **a** and α cells) to that of opaque **a** cells. Genes were filtered for greater than 2-fold expression change and clustered via average linkage (see Experimental Procedures). Upregulated genes are yellow and downregulated genes are blue. (B) Examples of genes regulated by both biofilm conditions, sexual biofilm conditions only, or *WOR1* overexpression biofilm conditions only. (C) Venn diagrams illustrate biofilm genes with a 2-fold or more change in expression relative to wild type opaque **a** cells. Overlaps between transcriptional profiles were significant as determined by the hypergeometric distribution (Supp. Table. 4).

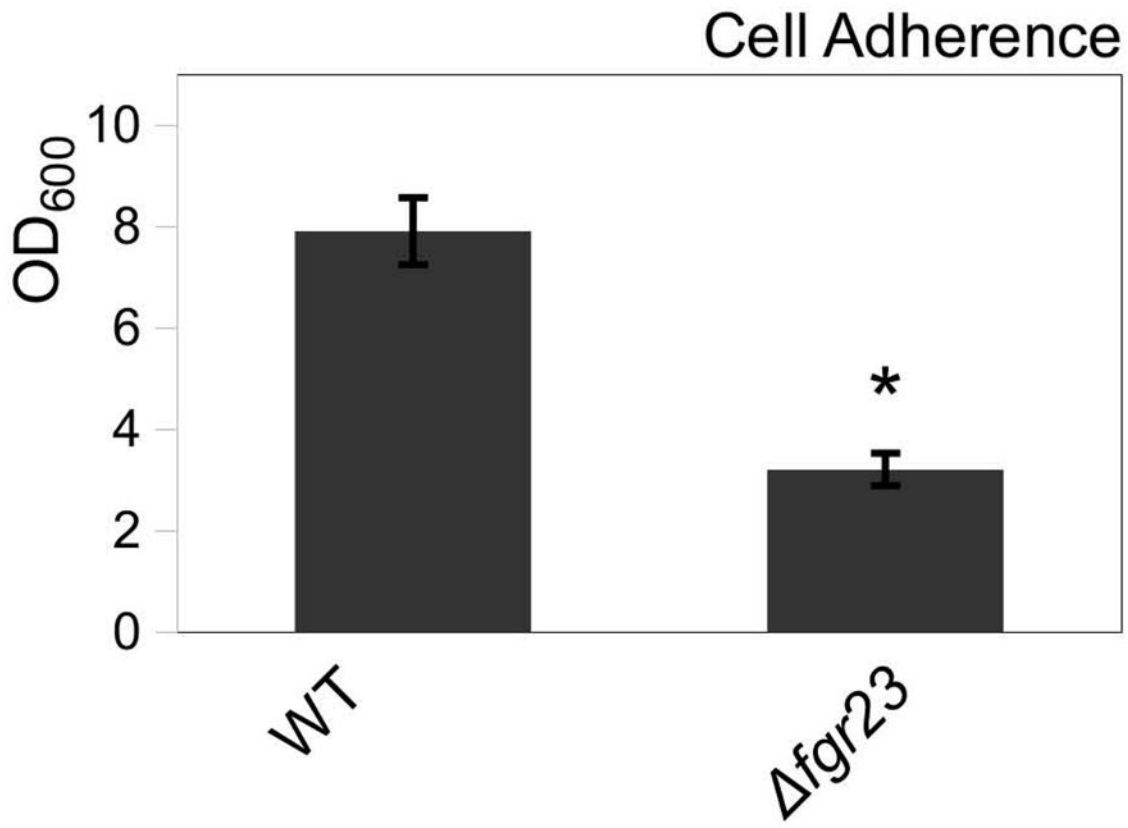

Figure 7.

The sexual agglutinin-related protein Fgr23 promotes sexual biofilm development. Deletion of *FGR23* decreases biofilm formation. Mean ± standard deviation (N≥4). * $p<0.05$. Mann-Whitney.