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Candida albicans biofilm growth and dispersal: contributions to pathogenesis

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The fungal species *Candida albicans* is most frequently associated with biofilm formation in immune-compromised and medically compromised patients, and it is now firmly established that biofilm formation represents a major virulence factor during candidiasis. A growing body of evidence has demonstrated that *C. albicans* biofilm development is a highly regulated and coordinated process, where adhesive interactions, morphogenetic conversions, and consortial behavior play significant roles. Cells within the biofilms are protected from environmental stresses including host immune defenses and antifungal treatment, which carries important clinical consequences for the treatment of biofilm-associated infections. Dispersal of cells from biofilms represents one of the hallmarks of the biofilm life-style, and in the case of *C. albicans* dispersed cells are responsible for candidemia and dissemination leading to the establishment of invasive disease.

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Introduction

Candida albicans remains the main etiological agent of candidiasis, the most common invasive fungal infection and now the third-to-fourth most frequent infection in hospitals worldwide [1,2]. A trait that greatly complicates treatment of these infections in an increasing number of patients is the ability of *C. albicans* to form an organized consortia of cells called biofilms, on indwelling medical devices [3]. Research on *C. albicans* biofilms is a little older than two decades; but the last few years have seen an increased interest by multiple groups of investigators that continue to contribute new aspects to our understanding of the biofilm life cycle and its clinical consequences. Here, we provide a summary of some of these major contributions, with emphasis on some of the most recent work on this topic.

Candida albicans biofilm formation

C. albicans is normally a harmless member of the native human microbiota. However, it can take advantage of immune-compromised and/or medically compromised patients and cause a variety of opportunistic infections, ranging from superficial dermal and mucosal infections to life-threatening systemic candidiasis [1]. It is now fully established that a majority of manifestations of candidiasis are associated with biofilm formation on the surface of biological or artificial surfaces [4,5]. Different biomaterials are able to support *C. albicans* biofilm formation and distressingly, the increased incidence of candidiasis has virtually paralleled the increasing use of a broad range of medical devices in the clinical practice. Biofilms are defined as highly organized attached microbial communities typically surrounded by a self-produced matrix of exopolymeric materials [6]. Biofilm formation complicates treatment and contributes to high morbidity and mortality rates, and as such represents one of the major virulence factors contributing to the pathogenesis of candidiasis [7•,8].

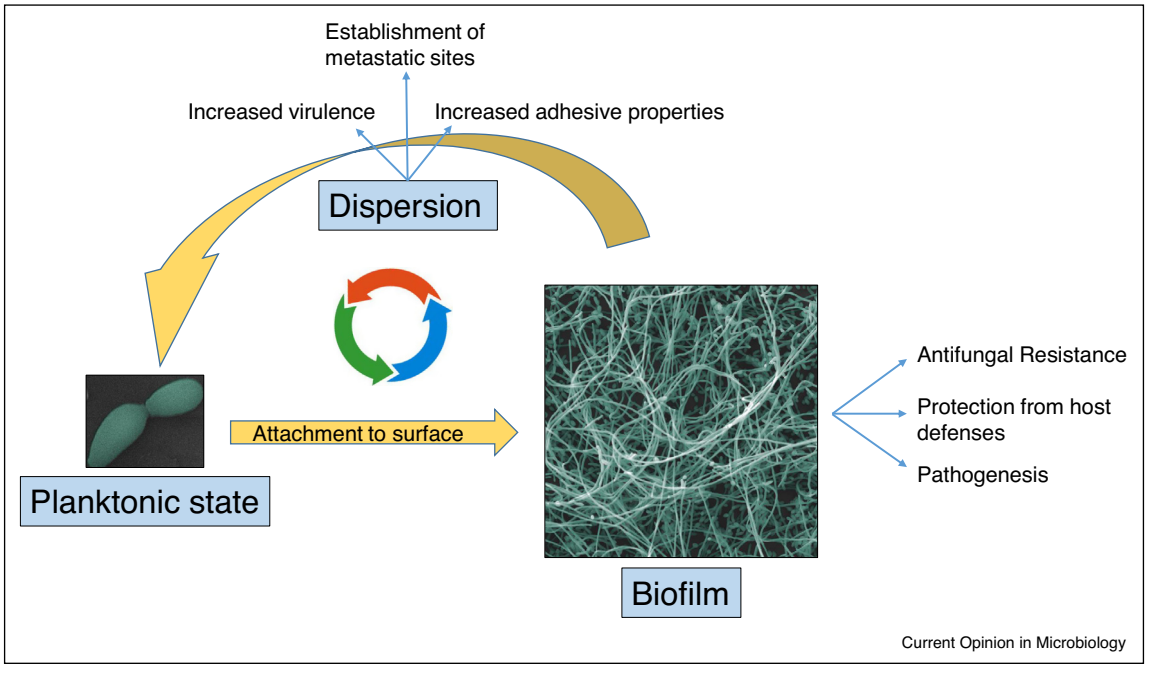
Overall, the *C. albicans* biofilm developmental process can be divided into four major phases: adherence, proliferation, maturation, and dispersal [9,10••]. In the early adherence phase, yeast cells attach to a material surface and form a basal layer that will anchor the biofilm to the surface. This is followed by a proliferation phase, which is characterized by the initiation of filamentation leading to the emergence of hyphal and pseudohyphal cells that continue to elongate during the entire biofilm developmental process forming a complicated network that contributes to the overall robustness of the biofilm. In the subsequent maturation phase, the hyphal scaffold become encased in a blanket of self-produced exopolymeric substances (EPS) that essentially act as an adhesive glue that holds the entire biofilm structure together. The

C. albicans EPS is composed of carbohydrates, proteins, lipids and eDNA which interact with each other conferring the matrix properties of an amalgam [11•,12]. As a part of this developmental process, *C. albicans* biofilms continuously release yeast cells of a unique elongated morphology that serve to seed new sites of infection. Dispersal stage ensures that the 'biofilm life-cycle' can be repeated all over again [13]. Under most experimental conditions, the entire process normally takes 24–48 hours, and a mature biofilm is typically several hundred micrometers in thickness. Overall, it is considered that this structural complexity represents the optimal spatial arrangement to facilitate the influx of nutrients, disposal of waste products and the establishment of microniches throughout the biofilm. Figure 1 shows the *C. albicans* transitions between the planktonic and biofilm states.

Most of the information on *C. albicans* biofilms has come from *in vitro* models. Although initial models were somewhat cumbersome and required expert handling and the use of specialized equipment [14••], the development of a 96-well microtiter plate model biofilm formation represented a significant step toward the simplification and standardization of *C. albicans* biofilm formation [15–17]. This technique involves the formation of multiple equivalent fungal biofilms on the bottom of wells of microtiter plates, coupled with some type of colorimetric read-out to allow for the estimation of the extent of biofilm formation or metabolic activity of cells within the biofilms. As such, the method is simple, flexible, relatively inexpensive, accurate, and robust; and because of its advantages, it has been subsequently adopted by many different groups around the world and democratized fungal biofilm research despite some inherent limitations. Other models involve the formation of *C. albicans* biofilms under conditions of flow, that more closely mimic physiological conditions within the human body [18]. More recently, a series of articles have described the development of nano-biofilm arrays, in which as many as one thousand equivalent miniaturized biofilms, each of approximately 30 nanoliters in volume can be formed on a single modified microscope slide, allowing for high throughput applications [19,20]. Notably, any and all these *in vitro* biofilm models reflect architectural features and properties of biofilms formed *in vivo*, in different animal models and also from those recovered from clinical samples, providing validation to these *in vitro* models [14••].

The entire process of biofilm formation is highly regulated at the molecular level. In the past decade molecular studies have begun to shed light on the signaling processes underlying the biofilm mode of growth in *C. albicans*. Early studies demonstrated a key role for morphogenetic transitions, adhesive interactions and quorum sensing in the formation of *C. albicans* biofilms [21,22]. Seminal work by the Mitchell group started to dissect the contributions of individual genes/proteins to biofilm formation and maintenance, leading to the identification of key transcriptional factors and adhesins involved in biofilm formation [9,23,24]. For example,

Figure 1



C. albicans transitions between the planktonic and biofilm life-styles and their major implications contributing to pathogenesis.

the *C. albicans* transcription factor mutants Δ*efg1* and Δ*tec1* are defective in biofilm formation due to their inability to filament. Similarly another transcription factor Bcr1 was identified as a major regulator of biofilm formation [25] due to its control of several hyphal adhesins including Als3 and Hwp1 whose complementary functions provide cohesiveness to the biofilm structure [25–27]. At the same time, different groups of investigators used powerful transcriptomic techniques to examine global patterns of gene expression in *C. albicans* biofilms as compared to their planktonic counterparts, which mostly highlighted the role of metabolism in biofilm growth [10••,28]. Subsequently, a seminal report by Nobile and colleagues revealed that a core network of nine interwoven transcription regulators (Bcr1, Brg1, Efg1, Ndt80, Rob1, Tec1, Flo8, Gal4 and Rfx2) are required for normal biofilm formation [29,30]. Together, these recently evolved regulators control the expression of about one thousand genes, representing approximately 15% of the entire *C. albicans* genome. Of note, several of these transcription factors also control the yeast-to-hyphae morphogenetic conversion, thereby establishing firmly that filamentation and biofilm growth are intimately linked.

Role of *C. albicans* biofilms in virulence

The NIH estimates that approximately 80% of infections in the United States are associated with a biofilm etiology, and this also holds true for *C. albicans* infections [4,5]. As mentioned before, the formation of biofilms by *C. albicans* carries notable clinical repercussions, contributing to higher mortality rates. Thus, it is now widely accepted that biofilm formation represents one of the main virulence traits associated with the pathogenesis of candidiasis [31•]. Biofilms provide a safe haven for fungal cells and can act as reservoirs for persistent sources of infections. From a clinical perspective, the two major consequences of biofilm formation negatively impacting the management of patients with these infections are the increased resistance of cells within the biofilms against antifungal therapy and their protection from host defenses [3,32•].

There are three main classes of antifungals used to treat candidiasis: azoles, polyenes and echinocandins [33]. *C. albicans* cells within a biofilm display high-levels of resistance to azoles and polyenes [33,34], with biofilms formed under flow conditions exhibiting even higher resistance [18]. Biofilms are intrinsically resistance to fluconazole and other azole derivatives. The anti-biofilm activity of polyenes occurs at high concentrations which generally are considered toxic and unsafe; although liposomal formulations show increased activity [35]. In contrast echinocandins, the newest class of antifungal agents targeting the cell wall component beta-1,3 glucans, display excellent activity against *C. albicans* biofilms at therapeutic concentrations, and used as first line therapy against these infections [33,35]. Multiple mechanisms contribute to the high levels of antifungal drug resistance exhibited by *C. albicans* biofilms, and for details, readers are referred to an excellent review on this topic [36]. Briefly, the biofilm extracellular matrix is a major contributor to resistance, sequestering antifungal molecules and preventing their penetration into the depths of the biofilm. Among other contributors to resistance are the increased cell density (i.e. safety in numbers), overexpression efflux pumps linked to drug resistance, changes in the sterol composition of the cell membrane, and presence of a subpopulation of persister cells that can tolerate high concentrations of antifungals [36].

Although much less is known about mechanisms of protection of *C. albicans* biofilm from host defenses, the last few years have seen an increasing number of articles devoted to this topic. Distinct components exposed on the surface of the biofilm matrix as compared to those on the fungal cell wall are likely responsible for differences in the interaction with pattern recognition receptors in host immune cells contributing to immune evasion [11•,32•]. In particular, cells within biofilms are protected from killing by neutrophils, macrophages and monocytes, which normally play important roles in the immune response against disseminated candidiasis [32•]. For example, compared to planktonic organisms, *C. albicans* biofilms inhibit the release of neutrophil extracellular traps (NETs) and impair the generation of reactive oxygen species (ROS) by neutrophils [37,38]. *C. albicans* biofilm formation also dampens macrophage migration, which is likely independent of the matrix and related to its physical structure [39]. Monocytes fail to phagocytose biofilm-associated *C. albicans* also leading to an altered cytokine profile, particularly the downregulation of TNF-α, a cytokine which facilitates phagocyte activation and which plays a key role in the protection against candidiasis [40].

Further evidence for the role that *C. albicans* biofilms play during infection comes from recent reports on the development of anti-virulence approaches for the treatment of candidiasis. Pierce *et al.* described a large-scale screening assay of chemical libraries in search for new small molecule compounds that inhibited *C. albicans* biofilm formation [41]. Several hits were identified belonging to a novel series of diazaspiro-decane structural analogs. Further characterization of the leading compound from this series confirmed that it did not affect growth of *C. albicans* (unlike conventional antifungals) but rather inhibited the ability of the fungus to form biofilms [41]. The fact that treatment with this anti-biofilm compound was effective against both oral and systemic candidiasis using animal models of infection provides corroboration of the role of biofilms in the pathogenesis of *C. albicans* infections. Similar results have also been reported for other compounds with the ability to inhibit *C. albicans* filamentation and biofilm formation [42,43••].

Dispersal of cells from biofilms

Once established, biofilms initiate or prolong infections by providing a safe sanctuary from which organisms can disperse and seed new infection sites. Recent reports have described that biofilm-dispersed cells arise mostly from the top-most hyphal layers of biofilms, and have a unique phenotype (elongated yeast cells) [13]. The frequency of dispersal is directly dependent on the carbon source and pH of growth media, wherein glucose induces higher frequencies of lateral yeast cells than alternative carbon sources. Importantly, in comparison to age-matched planktonic yeast cells, dispersed lateral yeast cells display enhanced adhesion to and damage of endothelial cells, increased filamentation and formation of denser biofilms, higher fluconazole resistance, and enhanced virulence in a murine model of hematogenously disseminated candidiasis [13]. RNAseq analysis on age-matched biofilm hyphae, dispersed cells, and planktonic yeast cells revealed that despite their yeast morphology, >60% of the differentially regulated genes in the dispersed cells were similar in expression to parent hyphae, making them a unique cell type. Consistent with their virulent phenotype, dispersed cells upregulate genes involved in adhesion (*ALS5*, *ALS6*, *ECM33*), drug resistance (*MDR1*, *QDR1*, *ERG* genes), nutrient acquisition (*ZRT1*, *ZRT2*, *ZAP1*), and pathogenesis (*SAP* genes), compared to age-matched planktonic yeast cells [44]. These results show that increased virulence of dispersed yeast cells is likely due to increased expression of genes associated with virulence. Reports by the Uppuluri group has demonstrated that the hyphae-to-yeast transition is paramount for dispersal; dispersed cells are yeast cells released from the lateral septal regions of hyphae present in the biofilm. These 'lateral yeast cells' are regulated by *PES1*, an essential gene conserved in all eukaryotes [13]. Negative genetic regulation of *PES1* expression either in an *in vitro* flow biofilm model or *in vivo*, in a catheter (implanted in the jugular vein of mice), blocked production of lateral yeast cells from biofilm hyphae and abrogated biofilm-associated disseminated candidiasis [13,44]. Thus, dispersed cells are virulent entities that ensure that a surface-bound drug resistant community of cells can further propagate into distal sites of infection. Approaches to inhibit the process of lateral yeast dispersal from drug resistant biofilms could serve as an alternative strategy to seal the biofilm reservoir and reduce dissemination.

Conclusions

Over the last approximately two decades, there has been an increasing appreciation of the role that biofilm formation plays in the biology and pathogenicity of *C. albicans*, and concomitantly, research on this topic has gained increasing momentum. Pioneering studies on the development of models for *C. albicans* biofilm formation and the description of their structural characteristics were followed by more in-depth mechanistic studies to understand biofilm development and its regulation at the molecular level. Likewise, the increased resistance of *C. albicans* biofilms against most conventional antifungal drugs has spurred new investigations in search for novel anti-biofilm agents targeting biofilms at various steps of its development. This drug-discovery process has been substantially facilitated by our increasing understanding of molecular mechanisms underpinning biofilm growth and drug resistance. A big hurdle yet to overcome is to identifying how the immune cells can be potentiated to combat biofilms. This may be just the tip of the iceberg as there are many other directions, such as the expansion of these studies to other non-*albicans Candida* species fully capable of biofilm development, and to the study of much more structurally complex and even more recalcitrant polymicrobial biofilms. It is our hope that this acquired knowledge will soon translate into the development of novel and/or alternative approaches to successfully combat the threat of *C. albicans* biofilm infections.

Conflict of interest statement

Nothing declared.

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