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Candida Biofilms: Development, Architecture, and Resistance

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Abstract

Intravascular device–related infections are often associated with biofilms (microbial communities encased within a polysaccharide-rich extracellular matrix) formed by pathogens on the surfaces of these devices. *Candida* species are the most common fungi isolated from catheter-, denture-, and voice prosthesis–associated infections and also are commonly isolated from contact lens–related infections (e.g., fungal keratitis). These biofilms exhibit decreased susceptibility to most antimicrobial agents, which contributes to the persistence of infection. Recent technological advances have facilitated the development of novel approaches to investigate the formation of biofilms and identify specific markers for biofilms. These studies have provided extensive knowledge of the effect of different variables, including growth time, nutrients, and physiological conditions, on biofilm formation, morphology, and architecture. In this article, we will focus on fungal biofilms (mainly *Candida* biofilms) and provide an update on the development, architecture, and resistance mechanisms of biofilms.

INTRODUCTION

The use of indwelling devices in current therapeutic practice is associated with hospital-acquired blood-stream and deep tissue infections (1). Transplantation medical procedures, immunosuppression, and prolonged intensive care unit stays have also increased the prevalence of nosocomial infections. Device-associated infections are commonly associated with the ability of bacteria and fungi to form biofilms, which are defined as communities of sessile organisms irreversibly associated with a surface, encased within a polysaccharide-rich extracellular matrix, and exhibiting enhanced resistance to antimicrobial drugs (2–5). Forming a biofilm provides the microbes protection from host immunity, environmental stresses due to contaminants, and nutritional depletion or imbalances, while being dangerous to human health due to biofilms' inherent robustness and elevated resistance.

Fungal infections are the fourth most common cause of nosocomial bloodstream infection (6), with *Candida* spp. being the most common fungi associated with these infections. Among *Candida* spp. *Candida albicans* is the most prevalent species causing both superficial and systemic disease (although infections due to non-*albicans* species are increasing). Even with current antifungal therapy, mortality associated with candidiasis can

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be as high as 50% in adults and up to 30% in children (7–10). In one of the earliest studies documenting the ability of *Candida* to form biofilms, Marrie and Costerton reported formation of *Candida parapsilosis* biofilms on vascular catheters (11). Initial studies also reported that *Candida* biofilms formed on different surfaces including Hickman catheters (12), soft contact lenses, ureteral stents (13), and corneas (14). Subsequent studies have demonstrated that *Candida* biofilms can form on a wide variety of indwelling medical devices including dentures, central venous catheters (CVCs), and urinary catheters.

Recent technological advances have facilitated the development of novel approaches to investigate the formation of biofilms and identify specific markers for biofilms. These studies have provided extensive knowledge of the effect of different variables, including growth time, nutrients, and physiological conditions, on biofilm formation, morphology, and architecture (15). In this chapter, we will focus on *Candida* biofilms (biofilms caused by *Aspergillus* are covered in reference 155) and provide an update on their development, architecture, and resistance mechanisms.

EXPERIMENTAL MODELS OF *CANDIDA* BIOFILMS

Microbial biofilms undergo multistep growth processes involving physical, chemical, and biological changes (16). Due to the versatility with which *Candida* biofilms can develop in human hosts, it is necessary to develop reproducible *in vitro* and *in vivo* models that could mimic these forms/situations. It is also necessary to develop models that can establish common and specific characteristics of *Candida* biofilm morphology. In this respect, various model systems have been studied to investigate the properties of microbial biofilms *in vitro* (17). These range from simple assays with catheter discs to more complex flow systems, such as the perfused biofilm fermenter or reactors and shear stress rotating disc systems (18, 19). Subsequent *in vitro* model systems have included forming biofilms on a variety of different plastics, microtiter plates, biofilm chips formed on glass slides, Calgary biofilm devices, microporous membrane cellulose filters, acrylic strips, voice prostheses, catheter discs, contact lenses, and tissue culture flasks (20–28). Although a variety of substrates support the formation of biofilms, those formed on clinically relevant substrates such as catheters, denture acrylic strips, voice prostheses, and contact lenses under physiological conditions are likely to be closer to the clinical setting than those formed on nonphysiologically relevant substrates.

Biofilm formation *in vitro* generally proceeds through three sequential steps: (i) pretreatment of the substrate, (ii) cell attachment, and (iii) colonization of cells and matrix formation. Various models have been evaluated to study detailed development, architecture, and morphology of biofilms (summarized in Tables 1 and 2). (These studies are described in greater detail in reference 156, and only a brief summary, relevant to *Candida* biofilms, is presented here.)

In vitro Models

In one of the first *in vitro* models of *Candida* biofilms, Hawser and Douglas (22) formed *C. albicans* biofilms on discs cut from a variety of catheters including latex urinary catheters, polyvinyl chloride CVCs, silicone elastomer-coated latex urinary Foley catheters, silicone

urinary Foley catheters, and polyurethane CVCs. These investigators quantified biofilm growth using a colorimetric assay based on reduction of a tetrazolium salt (3-[4, 5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide [MTT]) or incorporation of ^{3}H-leucine (22). This study reported an increase in MTT values and ^{3}H-leucine incorporation levels with the maturation of biofilms and showed that both quantification methods resulted in strong correlation with biofilm dry weight (22). An *in vitro* voice prosthesis biofilm model was described by Everaert et al. (27), who evaluated biofilm formation on argon plasma-treated silicone rubber voice prostheses.

Our group investigated the development and characterization of *C. albicans* biofilms formed on two common bioprosthetic materials: (i) silicone elastomer, a commonly used catheter material (20), and (ii) polymethylmethacrylate, used to form denture acrylic (21). Briefly, cells were adhered on these substrates and then transferred to the specific media for biofilms to mature (20, 21, 29). We also used various soft contact lenses to analyze differences in biofilm architecture (23). Measurement of biofilm growth was performed using two quantitative methods: (i) colorimetric assays that involved the reduction of 2,3-bis (2-methoxy-4-nitro-5-sulfophenyl)-5-[(phenyl amino) carbonyl-2H-tetrazolium hydroxide] (XTT) by mitochondrial dehydrogenase in the living cells into a colored water-soluble product measured spectrophometrically and (ii) dry weight determination, in which biofilms were scraped off the substrate surface and filtered through a preweighed membrane filter under vacuum (20–22). Our results showed that dry weight and XTT values increased with the formation of biofilms (20). The study showed that there was amorphous granular material covering yeast and hyphal forms and identified the developmental phases (20) associated with the biofilm growth: early (0 to 11 h), intermediate (~12 to 24 h), and mature phases (24 to 48 h) (20).

High-Throughput Models

To facilitate screening of compounds for their antibiofilm activity it is necessary to develop high-throughput biofilm models. Ramage et al. (30) used a microtiter plate model to assess the variability between *C. albicans* biofilms formed in independent wells of the same microtiter plate. All biofilms formed on the microtiter plates over a 24-h period displayed consistent metabolic activity (30). Our group developed a microtiter plate–based assay using catheter discs, in which biofilms are formed on catheter discs placed in the wells of a microtiter plate (31, 32). The advantage of this model is that biofilms are formed on actual catheter material instead of the plastic surface of a microtiter plate, which allows concomitant quantitative (XTT, dry weight) and microscopic (fluorescence, electron, confocal) evaluation of *Candida* biofilms at the same time (31, 32). Another microtiter plate model is the Calgary Biofilm Device model, developed by Ceri et al. (33) at the University of Calgary. This device has been used by several investigators in different studies, including evaluation of the ability of *Candida glabrata* to form biofilms (34), susceptibility of *Candida* biofilms to metal ions (35), interspecies variations (36), and identification of persister cells in *Candida* biofilms (37). More recently, Srinivasan et al. (38) developed a *C. albicans* biofilm chip microarray system (CaBChip), which comprises more than 700 independent and uniform nano-biofilms encapsulated in a collagen matrix and represents the first miniature biofilm model for *C. albicans*. Despite several-fold miniaturization, the

biofilms formed on the chip had similar phenotypic characteristics as *in vitro* biofilms, including a mixture of yeast, pseudohyphae, and hyphal cells, and a high level of antifungal drug resistance (38). The models represent exciting advances in the field and are likely to facilitate rapid and in-depth analysis of *Candida* biofilms and allow the identification of potential antibiofilm drugs.

In vivo Models

Several investigators have developed *in vivo* models to characterize and delineate the role of biofilms in animals (reviewed in depth in reference 156). In this regard, catheter-associated *Candida in vivo* biofilm models have been developed in rodents which provided information on biofilm architecture and antifungal resistance (39). These catheter-based *in vivo* biofilm models showed similar biofilm structures as seen using *in vitro* models after 24 h, with layers of yeast, pseudohyphae, and elongated hyphal cells embedded in an extracellular matrix (40). Our group developed a rabbit model of catheter-associated *C. albicans* biofilm infection (41) and showed that 7 days postinfection, quantitative catheter cultures consistently yielded >2 log CFU/catheter segment, which is considered the threshold for catheter-related infections. We also used a subcutaneous mouse model to evaluate the effect of coating a catheter substrate with amphogels (amphotericin B–based gel) on *Candida* biofilms (42) and demonstrated that the subcutaneous model has utility in studies evaluating catheter surface modification on the ability of *Candida* to form biofilms.

While the majority of biofilm models have focused on *Candida*, our group developed a murine model of contact lens–associated *Fusarium* keratitis (43, 44). This model was prompted by the association of fungal keratitis and biofilm noted in an outbreak of this disease in humans (45–47). *Fusarium*-infected mice had severe corneal opacification within 24 h, which progressed with unimpaired fungal growth in the cornea and with hyphae penetrating into the anterior chamber (43, 44).

Host Tissue–Associated *Candida* Biofilm Models

Biofilms formed on host surfaces are not well characterized, since tissue samples are sparse and not easily available (48). This gap was partially addressed in a mucosal model of oropharyngeal candidiasis *in situ* in mice, which demonstrated for the first time that epithelial cells, neutrophils, and commensal oral bacteria coexist within fungal biofilms formed on mouse tongue (48, 49). *C. albicans* can also form biofilms on the vaginal mucosa, with typical biofilm composition of yeast and hyphal cells embedded in an extracellular matrix; this vaginal biofilm model was replicated in immunocompetent estradiol-treated mice (50).

Development of these *in vitro* and *in vivo* models has allowed detailed investigation, microscopic evaluation, and gene/protein profiling of *Candida* biofilms. The availability of *in vivo* models is especially encouraging since this allows the conduct of studies aiming to elucidate host-pathogen interactions occurring on biofilms as they exist on bodily tissues.

BIOFILM MORPHOLOGY AND ARCHITECTURE

Candida biofilms formed *in vitro* comprise fungal cells embedded in a polysaccharide-rich extracellular matrix. When formed *in vivo* or in samples obtained from patients (e.g., used intravascular catheters, urinary catheters), these biofilms also contain host-derived biomolecules such as fibrinogen, dead cells, etc. In this regard, Lazarus et al. (51) reported catheter- and drug-induced occlusion in CVCs inserted into patients with malignancies before administration of intensive cytotoxic therapy; these CVC-associated occlusions (biofilms) were noted in Gram-positive and -negative bacteria (78%) and fungi (22%). Marrie and Costerton (11) performed scanning electron microscopy of *C. parapsilosis* biofilms formed on vascular catheters and reported *Candida* biofilm to contain fungal cells in a fibrous matrix resembling fibrin. Other investigators (12) reported similar morphology for *Candida* biofilms by scanning and transmission electron microscopy. Hawser and Douglas (22) used scanning electron microscopy to demonstrate that mature *C. albicans* biofilms (grown for 48 h) consisted of a dense network of yeasts, germ tubes, pseudohyphae, and hyphae, with extracellular polymeric material on the surfaces of some of these morphological forms.

Our group characterized the surface topography and three-dimensional architecture of *Candida* biofilms formed on denture strips and catheter discs (21, 52). Initially, scanning electron microscopy analyses of *C. albicans* biofilms formed on denture strips revealed a dense layer of coaggregating blastospores, and few hyphal elements, embedded in an extracellular, granular, polymeric matrix (21). Subsequent analyses of denture- and catheter-associated *Candida* biofilms with fluorescence microscopy and confocal microscopy revealed important differences in the surface topography and three-dimensional architecture of biofilms formed on these two substrates (52). Fluorescence microscopy showed that *C. albicans* biofilm formation on denture strips proceeds in three distinct developmental phases: early (≈0 to 11 h), intermediate (≈12 to 30 h), and maturation (≈38 to 72 h) phases. In the early phase, *C. albicans* cells grew as blastospores (yeast forms) adhering to the denture surface, which continued to grow as distinct colonies. In the intermediate phase, the fungal cells coaggregated into thick “tracks” due to growth along areas of surface irregularities and produced a noncellular, polysaccharide-rich “hazy” film covering the aggregating colonies. As the biofilms matured with time, the amount of extracellular material increased, until *C. albicans* communities were completely encased within this extracellular matrix. Biofilm formation on catheter (silicone elastomer) substrate exhibited similar phases, with one key difference: these biofilms had abundant hyphal elements. The difference in biofilm morphology was associated with the presence of a salivary conditioning film on denture biofilms, while the catheter biofilms contained a conditioning film of serum, a known inducer of filamentation in *C. albicans*.

Confocal microscopy analyses revealed a highly heterogeneous architecture of mature *C. albicans* biofilms in terms of the distribution of fungal cells and extracellular material. These analyses also underscored the key differences in architecture between the denture and catheter biofilms. Denture biofilms were 20 to 30 μm thick, comprised mostly yeast cells in confluent layers, and had irregular topography. In contrast, catheter biofilms were much thicker (up to 450 μm thick), with a 10- to 12-μm-thick basal layer of yeast cells overlaid

with a hypha-rich layer and uniform thickness. These differences could be attributed to differences in nutrient conditions and substrate properties for the two models.

FACTORS INFLUENCING BIOFILM FORMATION AND ARCHITECTURE

Biofilm formation is influenced by several host and *Candida*-derived variables, including fluid flow, nutrients, host receptor, and microbial products.

Fluid Flow

Physiological conditions including fluid flow at the infection site are important modulators of biofilm, since the flow of liquids can influence the nutrient exchange and structural integrity of biofilms (53–56). Efforts have been made to mimic these conditions *in vitro*, including mimicking the flow of saliva, blood, and urine, and the use of continuous flow cells to evaluate fungal biofilms. In this regard, Busscher et al. (57) investigated the ability of *C. albicans* and *Candida tropicalis* to form biofilms on silicone rubber voice prostheses with or without a salivary conditioning film in a parallel-plate flow chamber, and showed that biofilms formed under flow in the presence of salivary film tended to detach faster than those formed directly on the substrate. Other investigators used the parallel-plate flow chamber to evaluate formation of *Candida*-bacteria mixed biofilms on glass (58, 59) and acrylic (60). Zimmermann et al. (61) used the continuous flow culture to show that when tested under anaerobic conditions, fluconazole and voriconazole exhibit cidal activity, while under aerobic conditions, these agents were static against *Candida* biofilms. Our group investigated the effect of liquid flow shear on *Candida* biofilms using a rotating disc system (19), mimicking catheters placed intravenously that are exposed to shear stress caused by blood flow. Briefly, biofilms were formed on catheter discs and exposed to physiological levels of shear stress using a rotating disc system. Control biofilms were grown under conditions of no flow (19).

Tetrazolium assay and dry weight measurements were used to quantify metabolic activity and biofilm mass, respectively (19). Suci and Tyler (62) described an *in situ* method for assessment of the activity of chlorhexidine against *Candida* biofilms in a flow cell system by monitoring the kinetics of propidium iodide (PI) penetration into the cytoplasm of individual cells during dosing with chlorhexidine. This model allowed monitoring of the rate of PI penetration into the different subpopulations (yeast vs. hyphae) of the biofilm. Hawser et al. (63) showed that *Candida* biofilms formed under flow produced increased levels of extracellular matrix compared to those formed under static conditions. These results were confirmed in a subsequent study by the same group (64). Investigators have also used airflow models to evaluate voice prostheses, since obstruction of airflow is a major cause of early, premature replacement of these devices (65, 66).

Substrate

The role of substrate in modulating the ability of *Candida* to form biofilm has been demonstrated in several studies, which show that different substrates can greatly influence the architecture, morphology, and thickness of biofilms. Hawser and Douglas (22) evaluated various catheter materials and showed that biofilm formation by *C. albicans* was slightly

increased on latex or silicone elastomer ($P < 0.05$) compared with polyvinyl chloride but substantially decreased on polyurethane or 100% silicone ($P < 0.001$). Scanning electron microscopy demonstrated that after 48 h, *C. albicans* biofilms consisted of a dense network of yeasts, germ tubes, pseudohyphae, and hyphae; extracellular polymeric material was visible on the surfaces of some of these morphological forms. Our group investigated whether surface modifications of polyetherurethane (Elasthane 80A [E80A]), polycarbonateurethane, and poly(ethyleneterephthalate) can influence fungal biofilm formation (67). We found that biofilm formation by *C. albicans* was significantly reduced on 6PEO-E80A (by 78%) compared to biofilms formed on the nonmodified E80A (optical densities of 0.054 to 0.020 and 0.24 to 0.10, respectively; $P = 0.037$) (67). The total biomass of *Candida* biofilm formed on 6PEO-E80A was 74% lower than that on the unmodified E80A surface (0.46 to 0.15 versus 1.76 to 0.32 mg, respectively; $P = 0.003$). More recently, Estivill et al. (68) evaluated biofilm formation by 84 strains of five *Candida* species on three clinical materials and reported that all tested *Candida* strains were able to form biofilms and that all species showed greater biofilm formation capacity on Teflon, with the exception of *C. glabrata* which displayed higher biofilm formation capacity on polyvinyl chloride.

Taken together, these studies showed that the ability of *Candida* to form biofilms is greatly influenced by the type of material on which it grows and on the species and strain of *Candida*.

Nutrients

Nutrients in the growth media, including sugars, lipids, and serum, are crucial determinants of the biofilm-forming ability of *Candida*. Richards and Russell (69) investigated the effect of sucrose on the colonization of acrylic by *C. albicans* in pure and mixed culture in an artificial mouth and showed that the number of *Candida* cells was significantly increased on acrylic exposed to sucrose, while the number of salivary bacteria was unaffected by sucrose. In a separate study, the growth of *C. albicans* biofilms in medium containing 500 mM galactose or 50 mM glucose reached a maximum after 48 h and then declined; however, the cell yield was lower in low-glucose medium (22). Swindell et al. (70) determined the effect of parenteral lipid emulsion on *Candida* biofilms formed on medical catheter surfaces. Biofilms were formed on silicone-elastomer catheter discs and analyzed by scanning electron microscopy and confocal laser microscopy. Addition of lipid emulsion to a standard growth medium increased *C. albicans* biofilm production and resulted in changes in biofilm morphology and architecture. Furthermore, lipid emulsion induced germination and supported the growth of *C. albicans*. These findings may explain the increased risk of candidemia in patients receiving lipid emulsion via medical catheters. In a recent study, Samaranayake et al. (71) reported that human serum promotes *C. albicans* biofilm growth on silicone biomaterial and induces the expression of genes associated with adhesion (ALS3 and HWP1) and hydrolase-production (SAP, PLB1, and PLB2).

Species Variability

The ability to form biofilms may vary widely among and between strains of *Candida*. In this regard, in an early study, Branchini et al. (72) used electrophoretic karyotyping and pulsed-field gel electrophoresis to demonstrate genotypic variation and slime production among 31

isolates of *C. parapsilosis* obtained from patients with bloodstream or catheter infections. A total of 14 DNA subtypes were identified among the 31 isolates, of which 80% produced biofilms; biofilm-forming ability among the strains ranged from moderate to strong (67%) to weak (13%). Hawser and Douglas (22) compared biofilm formation by 15 different isolates of *C. albicans* and reported some correlation with pathogenicity: isolates of the less pathogenic *C. parapsilosis* (Glasgow), *Candida pseudotropicalis*, and *C. glabrata* formed significantly less biofilm ($P < 0.001$) than the more pathogenic *C. albicans*. Pfaller et al. (73) reported wide variability in the ability of clinical isolates of *C. parapsilosis* to form biofilms ("slime"). These investigators showed that 65% of the isolates tested produced biofilms (37% were moderately to strongly positive; 28% were weakly positive), and 35% did not form biofilms. A vast majority (83%) of the biofilm-forming isolates were blood and catheter isolates, suggesting that biofilm formation was closely associated with catheter-related bloodstream infections of *Candida*. Kuhn et al. (74) compared biofilms formed by *C. albicans* and *C. parapsilosis* on catheter surfaces using XTT and dry weight assays, followed by fluorescence microscopy and confocal scanning laser microscopy. These investigators reported significant differences in biofilm formation between invasive and noninvasive isolates of *C. albicans* ($P < 0.001$); *C. albicans* isolates produced more biofilm than *C. parapsilosis*, *C. glabrata*, and *C. tropicalis* isolates ($P < 0.001$ for all comparisons). Moreover, *C. albicans* biofilms consisted of a basal blastospore layer with a dense overlying matrix composed of exopolysaccharides and hyphae, while *C. parapsilosis* biofilms were comprised exclusively of clumped blastospores and had less volume than *C. albicans* biofilms. Unlike planktonically grown cells, *Candida* biofilms rapidly (within 6 h) developed fluconazole resistance (MIC > 128 μg/ml).

In a subsequent study, Silva et al. (75) characterized biofilms formed by three non-*albicans Candida* species (*C. parapsilosis*, *C. tropicalis*, and *C. glabrata*) recovered from different sources, using crystal violet staining. All non-*albicans Candida* species were able to form biofilms, although these were less extensive for *C. glabrata* than *C. parapsilosis* and *C. tropicalis*, and *C. parapsilosis* biofilm production was highly strain dependent. Scanning electron microscopy revealed that *C. parapsilosis* biofilm matrix had large amounts of carbohydrate with less protein. Conversely, matrix extracted from *C. tropicalis* biofilms had low amounts of carbohydrate and protein. Interestingly, *C. glabrata* biofilm matrix was high in both protein and carbohydrate content. Parahitiyawa et al. (36) used the Calgary Biofilm Device to evaluate biofilms formed by different *Candida* species and showed that *Candida* krusei developed the largest biofilm mass ($p < 0.05$) relative to *C. albicans*, *C. glabrata*, *Candida dubliniensis*, and *C. tropicalis*. These investigators also reported that *C. krusei* produced a thick multilayered biofilm of pseudohyphal forms embedded within the polymer matrix, whereas *C. albicans*, *C. dubliniensis*, and *C. tropicalis* biofilms consisted of clusters or chains of cells with sparse extracellular matrix material (34). Lattif et al. (76) characterized biofilm formation by 10 clinical isolates each of *C. parapsilosis*, *Candida orthopsilosis*, and *Candida metapsilosis* and reported that these three species formed biofilms to the same extent, as measured by XTT and biomass assays. However, strain-dependent variations in the metabolic activity of formed biofilms was noted for all three species tested. Scanning electron and confocal microscopy revealed that while the three species formed biofilms with similar topography and architecture, *C. metapsilosis* biofilms

showed a trend of lower biofilm thickness compared to *C. parapsilosis* and *C. orthopsilosis*. Estivill et al. (68) demonstrated similar trends of species-dependent biofilm formation by five different *Candida* species.

Taken together, these results demonstrated that biofilm-forming ability, structure, and matrix composition are highly species dependent. In general, *C. albicans* produces quantitatively larger and qualitatively more complex biofilms than other species.

Microbial Cohabitants

The ability of *Candida* to form biofilm is also affected by the presence of additional *Candida* species or of different bacterial cohabitants. In this regard, Holmes et al. (77) reported that *C. albicans* and *C. tropicalis*, two common oral fungi, bind to *Streptococcus gordonii*, while two other *Candida* species (*C. krusei* and *Candida kefyr*) do not. Moreover, there was a positive correlation between the ability of *Candida* to adhere to *S. gordonii* and adherence to experimental salivary pellicle. Whole saliva either stimulated or slightly inhibited adherence of *C. albicans* to *S. gordonii* depending on the streptococcal growth conditions. Reid et al. (78) showed that the ability of *Candida* to form biofilms on fibers and uroepithelial cells is affected by *Lactobacillus*. Fibers precoated with lactobacilli inhibited *Candida* adhesion by 0 to 67%, while lactobacilli exposure resulted in up to 91% displacement of preformed *C. albicans* biofilms. Experiments with uroepithelial cells also showed that the lactobacilli could significantly interfere with the adhesion of *Candida* to the cells, suggesting that members of the normal female urogenital flora might interfere with infections caused by *Candida*.

Webb et al. (79) showed that *S. gordonii* biofilms reduced the adhesion of *Candida* species to polystyrene. However, *Candida* species were able to coaggregate with *S. gordonii* in suspension, with one strain of *C. albicans* (GDH 2346, a denture stomatitis isolate) showing greater coaggregation than the other strains or species. Adam et al. (80) reported that extracellular polymer produced by *S. epidermidis* could inhibit fluconazole penetration in mixed *C. albicans*–bacterial biofilms. Conversely, the presence of *C. albicans* in a biofilm appeared to protect the slime-negative *Staphylococcus* against vancomycin. In a subsequent study, El-Azizi et al. (81) evaluated the interactions between *C. albicans* and 12 other species of *Candida* and bacteria in biofilms and reported reduced biofilm formation by *C. albicans* when the fungus was added to preformed biofilms of non-*albicans Candida* and bacteria. However, when *C. parapsilosis*, *Staphylococcus epidermidis* (a nonglycocalyx producer), or *Serratia marcescens* was added to preformed biofilms of *C. albicans*, the number of cells of the added microbes increased in the growing biofilms, demonstrating a dynamic interaction between *C. albicans* biofilms and other bacteria and fungi. In separate studies, Hogan et al. (82, 83) reported a pathogenic interaction between *Pseudomonas aeruginosa* and *C. albicans*. These investigators showed that *P. aeruginosa* formed a dense biofilm on *C. albicans* filaments and killed the fungus. In contrast, *P. aeruginosa* neither bound nor killed yeast-form *C. albicans*. Park et al. (84) recently reported that coculturing with bacteria decreased the biofilm-forming ability of *C. albicans*. van der Mei et al. (85) evaluated the ability of *C. albicans* and *C. tropicalis* to form biofilms on silicone voice prostheses in the absence and presence of various commensal bacterial strains and

Lactobacillus strains, and reported that biofilms consisting of combinations of *C. albicans* and a bacterial strain comprised significantly fewer viable organisms than combinations comprising *C. tropicalis*. Moreover, high percentages of *Candida* were found in biofilms grown in combination with lactobacilli.

The mechanisms underlying these interactions within *Candida* biofilms have been proposed to involve host products (e.g., salivary adhesins) as well as microbial proteins (e.g., *Candida* proteins and those produced by bacteria). Holmes et al. (86) reported that binding of *C. albicans* to *S. gordonii* involves multiple adhesin-receptor interactions, including the *S. gordonii cshA* and *csh*B genes (encoding high-molecular-mass cell surface polypeptides) and *ssp*A and *ssp*B genes (encoding antigen I/II salivary adhesins). Vilchez et al. (87) reported that *S. mutans* produces trans-2-decenoic acid (SDSF), a fatty acid signaling molecule, which inhibits *HWP1* expression in *C. albicans*, thus affecting fungal biofilm architecture. Studies have also shown that several *P. aeruginosa* virulence factors, including homoserine lactones and phenazine (e.g., pyocyanin), are involved in the inhibition of *Candida* biofilms (82, 83, 88–90).

These studies demonstrate that fungal-fungal and fungal-bacterial interactions play critical roles in modulating the ability of *Candida* to form biofilms. How these interactions relate to differences in microbial communities (bacteriome and mycobiome) within a biofilm is an area that has not been investigated and holds promise for future research efforts.

Candida Products

Studies performed using targeted gene disruptions, microarray-based transcriptomics, proteomics, and genomics have shown that several genes, proteins, and metabolites play critical roles in the maintenance of biofilm phenotype by *Candida* (see reference 157). In the first proteomic analysis of *Candida* biofilms, we identified alcohol dehydrogenase as one of the proteins that can modulate biofilms, by controlling the ethanolacetaldehyde conversion (91). In a subsequent study, we also performed proteomics analysis of the extracellular matrix of *Candida* biofilms (92). Initially, we compared five methods to isolate the matrix and showed that treatment with EDTA followed by ultrasonication was the optimal method to isolate this component of *Candida* biofilms. Proteomics analysis of biofilm matrix isolated using this optimized method revealed the presence of specific proteins (including glyceraldehyde 3-phosphate dehydrogenase and pyruvate kinase) in the biofilm matrix. Additional *Candida* genes implicated in biofilm formation include *ACE2* (93), *YWP1* (94), *HWP1* (95), *LL34* (*RIX7*) (96), *ALS3* (97, 98), *GAL10* (99), *VPS1* (100), *SUR7* (101), *GUP1* (102), *PEP12* (103), *TPK1/2* (104), *NRG1* (transcriptional repressor) and its target *BRG1* (GATA family transcription factor) (105), *UME6* (transcriptional regulator), *HGC1* (a cyclin-related protein), *SUN41* (a putative cell wall glycosidase), *EFG1* (106, 107), *STV1* and *VPH1* (Golgi/vacuolar subunits of vacuolar proton-translocating ATPase isoforms) (108), *CEK1* (map kinase) (109), *CDK8* (88), *BCR1* (110), *SPT20* (111), and *SAC1* (PIP phosphatase) (112). In addition, quorum sensing molecules (such as 3R-hydroxy-tetradecaenoic acid [3R-HTDE, a beta-oxidation metabolite of endogenously present linoleic acid] [113]), farnesol (114–117), and *cis*-2-dodecenoic acid (BDSF) (118) and metabolic processes (e.g., carbohydrate assimilation, amino acid metabolism, and intracellular

transport) (119) and glycolytic flux and hypoxia adaptation (120) have been suggested to play critical roles in *Candida* biofilm formation. The mechanism by which these genes and proteins modulate *Candida* biofilm formation and resistance phenotypes is currently being investigated.

ANTIFUNGAL SUSCEPTIBILITY PROFILE OF *CANDIDA* BIOFILMS

Candida biofilms are well documented to be resistant to commonly used antifungals, including azoles and polyenes (18, 121–124). Chandra et al. (21) evaluated the antifungal susceptibility of *Candida* biofilms formed on denture acrylic *in vitro* and showed that *C. albicans* biofilms exhibited resistance to amphotericin B, nystatin, chlorhexidine, and fluconazole. In contrast, planktonically grown *C. albicans* were susceptible to these agents. Separate studies have reported similar resistance profiles for *C. albicans* and *C. dubliniensis* biofilms formed in microtiter plates (30, 125). Kuhn et al. (126) evaluated *C. albicans* and *C. parapsilosis* biofilms formed on catheter discs and found that lipid formulations of amphotericin B and echinocandins showed activity against *Candida* biofilms. Confocal analyses revealed that treatment with voriconazole, caspofungin, and a lipid formulation of amphotericin B resulted in drug-specific morphological alterations. Bernhardt et al. (127) also reported that voriconazole stopped growth and colonization of *C. albicans* on cover slips in microtiter plates, and treated fungal cells exhibited short, swollen, deformed mycelia. Bachmann et al. (128) evaluated the *in vitro* activity of caspofungin against *C. albicans* biofilms and showed that this echinocandin displayed potent *in vitro* activity against *C. albicans* biofilms.

Scanning electron microscopy and confocal scanning laser microscopy indicated that caspofungin affected the cellular morphology and the metabolic status of cells within the biofilms. Coating of biomaterials with caspofungin had an inhibitory effect on subsequent biofilm development by *C. albicans*. Aminocandin, a newer echinocandin, has also been shown to exhibit anti-biofilm properties (129). Recently, Kaneko et al. (130) performed time-lapse microscopic observation of the effect of micafungin (an echinocandin) and fluconazole on *Candida* biofilms formed for up to 24 h on silicon disks in RPMI medium under flow (20 ml/h). These investigators showed that *Candida* biofilms grew at a uniform rate in the absence of drugs (17.2 ± 1.3 μm/h) and observed detachment of clusters of fungal cells from the hyphal tips in mature biofilms. Moreover, although neither drug eradicated biofilms, fluconazole exhibited an antibiofilm effect against early-phase (5-h grown) biofilms after 15 h of incubation. In contrast, micafungin suppressed biofilm growth within minutes after addition of the drug, with disruption of cells in the biofilms and release of undefined extracellular string-like structures from the burst hyphae.

Echinocandins, especially caspofungin, may also exhibit a paradoxical effect on *Candida* biofilms, defined as a resurgence of growth at drug concentrations above the MIC (131). These investigators reported that all *Candida* isolates (except *C. tropicalis*) displayed a paradoxical effect more frequently when grown as biofilms compared to planktonic cells. A paradoxical effect of echinocandin can also be discerned in the study by Kaneko et al. (130), who compared the antibiofilm activity of micafungin against early-phase biofilms after continued exposure for up to 24 h (described above). These investigators reported that after

15 h of incubation, micafungin-exposed biofilms exhibited some regrowth, compared to exposure for 5 min, when almost all the biofilm was inhibited. A paradoxical effect was associated with microscopic changes in cell morphology, manifested as the accumulation of enlarged, globose cells, suggesting drug-induced changes in cell wall composition as the mechanism underlying the paradoxical effect of echinocandins.

Several experimental agents are currently under investigation as potential antibiofilm drugs for *Candida* biofilms. These include chlorhexidine, sodium hypochlorite, zosteric acid, filastatin, EDTA/ethanol catheter lock solutions, gentian violet, and essential oils (62, 79, 132–142). In addition, physical interventions such as low-level laser (143), photodynamic therapy (144–147), and antimicrobial coating of catheters (148–151) have also been proposed as possible therapeutic alternatives. More detailed investigations are warranted to determine the efficacy of these agents against *Candida* biofilms.

MECHANISMS OF RESISTANCE OF *CANDIDA* BIOFILMS

The development of various models has allowed detailed evaluation and understanding of the mechanisms underlying *C. albicans* biofilm resistance. These methods include studying alterations in drug targets involving changes in membrane sterol, membrane localized drug efflux pump assays at the functional and transcriptional level, and reduced or limited drug penetration through biofilms.

The cellular target for azoles is a 14-α demethylase enzyme involved in the ergosterol biosynthetic pathway. Alterations in sterol composition are linked to antifungal resistance. Our group for the first time developed methods involving isolation of membrane sterols from biofilms (20). Briefly, total membrane sterols were isolated from biofilms and planktonic cells and were analyzed by gas liquid chromatography (20). These studies show that the ergosterol levels of biofilms grown to the intermediate and mature phases were reduced by 41 and 50%, respectively, compared to early-phase *C. albicans* biofilm. These results showed that the level of sterols is modulated during *C. albicans* biofilm formation and suggested that such modulation may contribute to drug resistance in a phase-specific manner (20). We also standardized an assay based on the efflux of Rhodamine 123 (Rh123), a fluorescent substrate for drug efflux proteins, to evaluate the functionality of efflux pump proteins (CDR/MDR proteins). Resistant cells over-expressing functional efflux pumps do not retain Rh123, while susceptible cells, which lack or have a low number of these pumps, retain the fluorescent dye, which is quantified by fluorescence measurements (152). Our results showed that in early-phase biofilms, efflux pumps contributed to antifungal resistance, while in mature-phase biofilms, resistance was associated with changes in the levels of ergosterol biosynthesis intermediates (152). The role of efflux pumps in biofilm-associated resistance was confirmed in a separate study by Mateus et al. (153), who evaluated efflux pump activity at the transcriptional level and showed that adherence of *C. albicans* to silicone induces immediate enhanced tolerance to fluconazole and that expression of *MDR1* and *CDR1* genes was significantly lower in daughter cells from 48-h biofilms than in firmly adherent cells (2 h after attachment), suggesting that efflux pump expression in adherent cultures is transient.

Next, our group investigated whether drug binding/penetration plays a role in the resistance of *C. albicans* biofilms against fluconazole. We performed preliminary studies by using equilibrium dialysis and diffusion bioassay methods (154). Briefly, the ability of fluconazole to bind/penetrate *Candida* biofilms formed on cellulose membrane was determined by using equilibrium dialysis equipment, which consists of two chambers (1 ml volume each) separated by the membrane. To form biofilm on the membrane, a fungal cell suspension (1×10^7 cells in yeast nitrogen base media) was added to chamber 1; the other chamber was filled with 1 ml yeast nitrogen base media, and the apparatus was incubated at 37°C for 48 h (154). After biofilm formation on the membrane, fluconazole (4, 64, 256, or 1,024 μg/ml) was added to chamber 1 and allowed to equilibrate for 48 h, and the amount of free drug in each chamber was determined as inhibitory zones using a diffusion bioassay (154). At a concentration of 64 μg/ml, fluconazole was equally distributed in the two chambers of the equilibrium dialyzer, indicating that the drug freely penetrated. In contrast, when *C. albicans* biofilm was incubated with a higher concentration of fluconazole (256 μg/ml or 1,024 μg/ml), the free drug equilibrated between chambers 1 and 2 accounted for 200 μg/ml, while 56 μg/ml fluconazole was bound to the biofilm. Furthermore, incubation of *Candida* biofilm with 1,024 μg/ml fluconazole also resulted in binding of 56 μg/ml of the drug, indicating saturation of biofilm at high concentrations. These studies showed that at clinically relevant low concentrations, fluconazole did not bind to the biofilm, suggesting that drug binding/penetration does not play a major role in azole resistance of *C. albicans* biofilms (154).

CONCLUSION

Recent advances have resulted in the development of an array of new tools and techniques to analyze *Candida* biofilms at the morphological, physiological, biochemical, and molecular levels, providing in-depth insight into their biology and pathogenesis. This new knowledge will fuel future investigations that are likely to lead to better management of diseases associated with fungal biofilms. Finally, the findings that fungi-fungi and fungi-bacteria affect each other in a mixed biofilm environment point to the need to understand how biofilms are influenced and the role of these interactions as components of microbial communities such as the mycobiome and bacteriome.

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TABLE 1

Summary of *in vitro Candida*-associated biofilm models

In *vitro* models	Substrate	Features
Plastic/microtiter plates	Polystyrene, flat-bottom 96-well plates, plastic slides	Biofilm for 96 strains/species can be tested at one time
Calgary Biofilm Device (CBD)	96-well polystyrene pegs/plates	CBD is a useful, simple, low-cost miniature device that has utility for parallel study of *Candida* biofilms and for elucidating factors modulating this phenomenon
Microporous membrane filters	Cellulose	Useful model to study antimycotic perfusion through biofilms and complex interactions between biofilm-antifungal interphase
Voice prostheses	Silicone rubber	Mimic clinical conditions
Catheters	Latex urinary catheters, polyvinyl chloride, CVCs, silicone elastomer-coated latex urinary Foley catheters, silicone urinary Foley catheters, and polyurethane CVCs; silicone elastomers	Clinically relevant substrates; mimic intravascular catheter-associated infections
Denture acrylic strips	Polymethylmethacrylate, acrylic resins	Mimic denture stomatitis, oral clinical conditions
Contact lenses	Lotrafilcon A, etafilcon A galyfilcon A, balafilcon A, alphafilcon A	Mimic keratitis and other eye-associated clinical conditions
Flow system biofilm models	Glass microfermentors	Provide a continuous flow of media or fluid, mimicking the physiological conditions present at the infection site (e.g., mimicking the flow of saliva, blood, or urine). Such flow of liquids can influence nutrient exchange and the structural integrity of biofilms
High-throughput biofilm models utilizing biofilm chip system (CaBChip)	Microarray platform with nano-biofilms encapsulated in a collagen matrix	Miniaturization and automation of chip cut reagent use and analysis time; minimize labor-intensive steps and reduces assay costs. Also accelerate the antifungal drug discovery process by enabling rapid, convenient, and inexpensive screening of hundreds to thousands of compounds simultaneously

TABLE 2

Summary of *in vivo Candida*-associated biofilm models

***In vivo* models**	**Animal species**	**Features**
Catheter-associated *in vivo* models, models utilizing amphogel coating on catheters and subcutaneous catheter models	Rat, mouse, rabbit	Rat and mouse models have advantage over rabbit models because they have a relatively low cost in setting, are easy to handle, and mimic the clinical conditions of rabbit models
Denture-associated models	Rat	Low cost, mimic clinical conditions
Contact lens *in vivo* models	Mouse	Low cost and clinically relevant
Models using biotic surfaces such as oral cavity, oropharyngeal mucosa, tongue, vaginal mucosa	Mouse	Low setting cost, mimic clinical conditions