ABSTRACT During infection, fungi frequently transition to a biofilm lifestyle, proliferating as communities of surface-adherent aggregates of cells. Phenotypically, cells in a biofilm are distinct from free-floating cells. Their high tolerance of antifungals and ability to withstand host defenses are two characteristics that foster resilience. Biofilm infections are particularly difficult to eradicate, and most available antifungals have minimal activity. Therefore, the discovery of novel compounds and innovative strategies to treat fungal biofilms is of great interest. Although many fungi have been observed to form biofilms, the most well-studied is Candida albicans. Animal models have been developed to simulate common Candida device-associated infections, including those involving vascular catheters, dentures, urinary catheters, and subcutaneous implants. Models have also reproduced the most common mucosal biofilm infections: oropharyngeal and vaginal candidiasis. These models incorporate the anatomical site, immune components, and fluid dynamics of clinical niches and have been instrumental in the study of drug resistance and investigation of novel therapies. This chapter describes the significance of fungal biofilm infections, the animal models developed for biofilm study, and how these models have contributed to the development of new strategies for the eradication of fungal biofilm infections.

SIGNIFICANCE OF FUNGAL BIOFILMS IN INFECTION

Many fungal and bacterial pathogens build biofilms, establishing resilient communities on a variety of clinical surfaces (1, 2). Biofilm formation has become increasingly appreciated as one of the most common modes of growth. Medically, these adherent conglomerates of cells pose a serious obstacle for the treatment of infection. Compared to nonbiofilm, planktonic cells, they are extraordinarily tolerant to anti-infective therapies and resist killing by host defenses (3). Biofilm formation has been well described for Candida albicans, the most common fungal pathogen in the hospital setting (4, 5). More recently, the majority of clinically encountered fungi have been shown to produce biofilms. This group includes filamentous fungi (Aspergillus, Fusarium, zygomycetes), Pneumocystis, and yeasts (Blastoschizomyces, Saccharomyces, Malassezia, Trichosporon, Cryptococcus, and numerous Candida spp.) (Table 1) (6–16).

One of the distinguishing traits of biofilm communities is their ability to adhere to a surface. In the medical setting, indwelling devices such as catheters provide an ideal niche for biofilm formation (1, 17). As technology advances, the use of medical devices continues to escalate. At least 35 million devices are implanted yearly in the United States alone, and the majority of hospital-acquired infections are device-associated (18). Many types of devices are at risk for biofilm infection, including catheters, dentures, implants, pacemakers, artificial heart valves, and central nervous system shunts (1, 18). Biofilm infections may be catastrophic, resulting...
TABLE 1 Medically important fungi forming biofilms

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<tr>
<td>Aspergillus</td>
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<td>Blastoschizomyces</td>
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<td>Candida</td>
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<td>Cryptococcus</td>
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<tr>
<td>Fusarium</td>
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<td>Pneumocystis</td>
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<td>Trichosporon</td>
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<td>Zygomyctes</td>
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in device malfunction or life-threatening, systemic infection (1). Candidiasis in the hospital setting usually involves biofilm infection of a medical device. Candida spp. are the fourth most common nosocomial bloodstream pathogens and the third most common urinary tract pathogens (19–22). While biofilm infection was initially described for C. albicans, the majority of Candida spp., including Candida dubliniensis, Candida glabrata, Candida krusei, Candida tropicalis, and Candida parapsilosis, have now been shown to cause biofilm infections (23).

Mucosal candidiasis is widespread. Vaginal candidiasis affects approximately 30 to 50% of women, and many suffer recurrent infections (24). The clinical relevance of biofilms on biotic surfaces has become increasingly evident, and mucosal biofilms have been described for Candida. In an animal model of oropharyngeal candidiasis, Candida forms a biofilm of yeast, hyphae, and commensal bacteria within the epithelial surface (25). Candida biofilms growing on the vaginal epithelial lining similarly demonstrate a typical biofilm architecture with adherent cells embedded in an extracellular matrix (26). Mucosal biofilms appear to have many similarities to biofilms growing on abiotic surfaces, including sessile growth, protection from environmental factors, and variable access to nutrients (27). However, biofilms on a mucosal surface participate in a dynamic interaction with the adjacent epithelial lining. The host epithelial lining may deliver immune component, nutrients, and antifungal components. Therefore, the basal aspect of a mucosal biofilm would be expected to be exposed to a vastly different environment when compared to a biofilm on an abiotic device surface.

Like Candida, other fungal pathogens, including Cryptococcus and Aspergillus, have been found in biofilms adherent to abiotic device surfaces (15, 28). However, for Aspergillus, the biofilm mode of growth is usually observed in the absence of a foreign body. Even in the absence of a device, Aspergillus has been shown to proliferate as a cellular aggregate encased in extracellular matrix with properties similar to device-associated biofilms (28). Clinical examples of these non-device associated biofilms include fungal sinusitis, pulmonary aspergillosis, and aspergilloma (7). Because the biofilm lifestyle of Aspergillus is a relatively new field of investigation, further investigations are needed to explore this growth mode.

FUNGAL BIOFILM TRAITS

Structure

Fungal biofilms are comprised of adherent cells covered by an extracellular polymeric matrix. The process of fungal biofilm formation in vitro was initially described for C. albicans in three main stages (29). First, during early biofilm formation, Candida cells adhere to the biofilm substrate. For C. albicans, germ tube formation may be elicited. During the intermediate biofilm formation stage, the extracellular matrix begins to appear and covers proliferating fungal colonies. Finally, mature biofilm formation is marked by extracellular matrix encasing the adherent yeast and developing hyphae. It is now recognized that dispersion is a key component of the dynamic nature of biofilms (30). Release of cells from biofilms is a regulated process by which organisms can disseminate throughout the host and establish new sites of infection. This developmental process appears to hold true for in vivo Candida biofilms (31, 32).

For Aspergillus, aerial, static conditions promote biofilm formation (33). Under these conditions, Aspergillus fumigatus grows as a cohesive mycelial structure. The cells become progressively encased in a hydrophobic extracellular material over time. Ultrastructure analysis reveals air channels embedded within the conglomerate (33). These multicellular communities, surrounded by acellular matrix material, also develop in vivo in a murine model of pulmonary aspergilloma and invasive aspergillosis (7).

Tolerance to Antifungals

Biofilms are notoriously difficult to treat in the clinical setting, and their physical removal is often required for eradication of infection (34). Available antifungal medications are seldom effective, given the high tolerance of biofilms to these commonly used anti-infective therapies. Candida biofilms proliferate in the face of antifungal concentrations up to 1,000-fold higher than those needed to inhibit nonbiofilm, planktonic cells (29, 35–37).

The biofilm lifestyle of C. albicans is associated with resistance to available drug classes compared to activity
against planktonic cells (29, 35, 38–42). Resistance to the azoles (fluconazole, itraconazole, voriconazole, posaconazole) is particularly pronounced, while the echinocandins (caspofungin, micafungin, anidulafungin) and liposomal amphotericin are more effective. Various mechanisms have been shown to contribute to this resistant phenotype, including the production of an extracellular matrix, an increase in efflux pump activity, alteration of sterols, production of resistant “persistor cells,” activation of stress responses, and an increase in cell density (40, 43–50). Biofilms formed by *Aspergillus* and *Cryptococcus* also display this multidrug resistance (8, 33, 51, 52). For *Aspergillus* biofilms, antifungal tolerance has been linked to the presence of an extracellular matrix, as well as an increase in efflux pump activity (52–54). Few investigations have examined the mechanisms underlying the antifungal resistance of *Cryptococcus* biofilms, but this phenotype has been correlated with the production of melanin (51).

### Immune Resistance

In addition to the well-described drug resistance, biofilm growth also appears to afford protection from the host immune response (55–58). Compared to planktonic cells, both neutrophils and mononuclear cells are less effective in killing *Candida* biofilm cells. Mononuclear cells become entrapped in biofilms but do not efficiently activate or phagocytize fungal cells (57, 59). Neutrophils have impaired function against both *C. albicans* and *C. parapsilosis* biofilms (55, 56, 58). Although most studies involving the leukocyte response to fungal biofilms have been limited to *in vitro* studies, the phenotype appears clinically relevant. An intact immune response is not sufficient to clear *Candida* biofilms. When coupled with antifungal therapy, improved clinical outcomes are observed when *Candida*-infected medical devices are removed (34, 60). Studies examining the innate immune response to *Candida neoformans* biofilms also demonstrated a resistant phenotype. *In vitro*, these biofilms are more tolerant of defensins and oxidative stress than are planktonic cells (61).

### HOST FACTORS INFLUENCING FUNGAL BIOFILMS

Fungal biofilms form in a variety of clinical niches. These sites of infection can vary quite significantly with regard to available nutrients, flow conditions, immune components, pH, and the substrate for cell adhesion and initiation of biofilm growth (Fig. 1). Each of these factors is likely to influence the biofilm properties and structure, as discussed below. Models most closely mimicking the clinical niche are necessary to best reproduce the host environmental conditions and ultimately a clinical biofilm infection.

#### Flow Conditions

One of the greatest environmental differences among the sites of common fungal biofilms is the flow conditions. For example, *Candida* can form a biofilm in the face of a low rate of salivary flow (denture stomatitis), a rapid current of blood (endocarditis), or an intermittent flow (vascular and urinary catheter infection) (18). The Douglas group investigated the influence of flow conditions on *C. albicans* biofilm architecture using *in vitro* models (62, 63). Compared to biofilms grown in static conditions, those propagated in a continuous flow device were encased in a higher concentration of extracellular matrix. The continuous-flow biofilms also exhibited increased resistance to antifungals, including amphotericin B and fluconazole. Investigation of continuous flow by an independent laboratory confirmed the drug resistance phenotype, as well as the altered biofilm structure (64). Biofilms formed in the flow

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**FIGURE 1** Host factors influence fungal biofilm formation. This schematic depicts host conditions that may impact fungal biofilm formation and architecture. *In vivo* animal models can closely mimic biofilm infection niches, incorporating many of these host and environmental conditions. doi:10.1128/microbiolspec.MB-0008-2014.f1
environment were more dense and compact. One might expect this architectural change to greatly impact other aspects of biofilm physiology, such as the availability of oxygen and nutrients.

As described above, *Aspergillus* has been shown to form biofilms during invasive pulmonary aspergillosis and pulmonary aspergilloma (7). In the lung, these biofilms proliferate in a static, aerial environment. Unlike *Candida* biofilms, the extracellular matrix production is mostly supported by these low flow conditions (33). The variability in key properties, including matrix production and drug resistance, of biofilms formed under a variety of flow conditions points to the importance of using models that best fit a particular physiologic niche.

**Substrates and Conditioning**

One of the most influential factors for biofilm initiation is the substrate for adherence (4, 65). Although many materials have been shown to support biofilm formation, the topography and hydrophobicity of the substrate may greatly impact fungal adherence. Indwelling medical devices are often designed to resist microbial adherence. Compared to other plastics, these materials, such as silicone, may even require a preconditioning, or protein coating, for robust biofilm formation in vitro (66, 67). However, in vivo, medical devices are rapidly conditioned with host factors from the surrounding fluids, such as blood, saliva, urine, or other fluids (68–72). Several of the factors that may coat various devices, influencing adherence and biofilm formation, include fibrinogen, fibronectin, vitronectin, thrombospondin-1, albumin, and von Willebrand factor (68–72). Because the concentrations of these substances differ among clinical niche sites, in vivo models best account for the conditioning of medical devices prior to biofilm initiation. When considering mucosal biofilms, substrate representation becomes even more complex. Here, the epithelial layer of cells provides a surface for microbial adhesion which also involves receptor-ligand interactions (27).

**Nutrient Composition**

Biofilm niche sites vary greatly with respect to the availability of nutrients. For example, blood is a fairly nutrient-rich environment, while urine has a lower abundance of sugars and proteins. In addition, the conditions may be altered by both illness and diet. Untreated diabetes mellitus raises the glucose content throughout the host, while a diet high in sugar primarily raises the glucose content in the oral cavity. The carbon source (galactose or glucose) and abundance can greatly influence biofilm integrity for both *C. albicans* and *C. neoformans* (65, 73). Another factor shown to influence *Candida* biofilm formation is the concentration of metal ions (Ni²⁺, Fe³⁺, Cr³⁺) (74). These variables can impact the rate of biofilm growth, the production of extracellular matrix, and the strength of the biofilm. Animal models of biofilm infection which utilize an equivalent anatomical site provide the ideal composition of nutrients and minerals to mimic patient biofilm infections.

**Host Immune Components**

Mounting evidence suggests a complex interaction between host immune cells and fungal biofilms. For example, leukocytes, important for controlling fungal infections, have also been shown to promote biofilm growth (59). *In vitro*, *C. albicans* biofilms were observed to proliferate in response to a soluble factor released by mononuclear peripheral blood cells. Ultimately, mononuclear cells became entangled within the basal level of a *C. albicans* biofilm and were not able to phagocytize the biofilm cells. When examining oral mucosal *Candida* biofilms, Dongari-Bagtzoglou et al. observed the migration of neutrophils throughout the biofilm (25). Host immune cells appear to incorporate into biofilm, and even augment biofilm growth, but are usually unable to contain the infection. To understand how the immune system impacts the biofilm lifestyle, models encompassing immune components at the site of infection are optimal.

**IN VIVO MODELS OF FUNGAL BIOFILMS AND DRUG DISCOVERY**

Animal models best integrate the influence of host factors on the formation of biofilms and acquisition of their phenotypic traits. Utilization of animal models incorporates not only the influence of the immune system, but also niche-specific factors, such as the flow conditions, nutrients in the environment, and the substrate or surface of adherence. Because *Candida* has served as a model organism for fungal biofilm infection in this arena, models involving this pathogen will be much of the focus of discussion in this article (Table 2 and Fig. 2).

**Vascular Catheter Model**

Perhaps the most commonly used animal model for *in vivo* biofilm study is the venous catheter model. This model has been adapted for use in a rat, a rabbit, and a mouse and has been instrumental for examining the
efficacy of antifungals against biofilms formed in vivo (31, 75, 76). As a close mimic of one of the most common clinical biofilm infections, biofilms on the luminal catheter surface are exposed to host conditions, flow, serum proteins, and immune components. The model involves surgical vascular catheter insertion (often jugular vein) followed by subcutaneous tunneling and securing with a protective device. Performing the procedure prior to luminal inoculation allows for a period for host protein conditioning of the device surface. The influence of anti-infectives on biofilm growth can be assessed following systemic administration of drug or by instilling in the lumen as a lock therapy. Common techniques include microscopy for evaluation of biofilm extent and architecture or viable burden determination (Fig. 3). Results of these studies have corroborated the multidrug-resistant phenotype of in vitro Candida biofilms and the need for discovery of new strategies and drugs to treat these infections (31).

One approach to circumvent the drug tolerance of biofilms is to directly administer antifungal in the form

<table>
<thead>
<tr>
<th>TABLE 2</th>
<th>Animal models of Candida biofilm infection</th>
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<tbody>
<tr>
<td>Central venous catheter</td>
<td>Rat (31)</td>
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<tr>
<td></td>
<td>Rabbit (75)</td>
</tr>
<tr>
<td></td>
<td>Mouse (76)</td>
</tr>
<tr>
<td>Urinary catheter</td>
<td>Mouse (98)</td>
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<tr>
<td>Subcutaneous implant</td>
<td>Mouse (93)</td>
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<tr>
<td></td>
<td>Rat (94)</td>
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<tr>
<td>Denture stomatitis</td>
<td>Rat (32, 87)</td>
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<tr>
<td>Oral mucosal</td>
<td>Mouse (25)</td>
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<tr>
<td>Vaginal mucosal</td>
<td>Mouse (26)</td>
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of lock therapy (75, 77–79) (Table 3). By avoiding the majority of systemic toxicities, significantly higher drug doses may be safely delivered. Vascular catheter animal models have been valuable for analyzing the efficacy of various antifungal lock therapies against Candida biofilms in vivo. When instilled in the lumen of C. albicans–infected catheters, several lock therapies were found to successfully treat the biofilm infections. Of the clinically available antifungals, the more efficacious solutions have included liposomal amphotericin B (10 mg/ml), amphotericin B lipid complex (5 mg/ml), and caspofungin (6.67 mg/ml) (75, 77–79). As might be predicted by in vitro biofilm susceptibility studies, the azole drugs and lower doses of echinocandin drugs have significantly less activity against vascular catheter biofilms and are not ideal for catheter lock therapy (75, 76). One concern regarding the use of available antifungals for lock therapy is the potential for fostering a resistance-promoting environment. As a method to avoid this possibility, studies have also explored the use of alternative agents such as biocides. Encouraging results have been observed in vitro for ethanol, ethylenediaminetetraacetic acid (EDTA), and high-dose minocycline (3 mg/ml) lock solutions (80–82).

A second tactic to overcome the profound antifungal tolerance of biofilms is combination drug therapy. The rat vascular catheter biofilm infection model has successfully been used to evaluate the in vivo efficacy of several combination therapy lock solutions (48, 49). These studies have explored the impact of adding agents targeting cellular stress responses onazole drug resistance. Uppuluri et al. demonstrated the efficacy of combining calcineurin inhibitors and fluconazole for lock therapy treatment of C. albicans biofilms (48). An agent inhibiting the calcineurin pathway (tacrolimus) was found to augment the activity of fluconazole against C. albicans catheter biofilms. Subsequent investigations suggest that calcineurin inhibitors similarly potentiate the activity of agents in other drug classes, including the echinocandins and amphotericin B (83). Robbins et al. also used a rat venous catheter model to test the efficacy of combination lock therapy (49). Combining an inhibitor of the Hsp90 pathway (17-AAG) with fluconazole improved the activity against C. albicans biofilms. The mechanism of this action is thought to involve a decrease in biofilm extracellular matrix, limiting the capacity of the matrix to sequester antifungal.

**Denture Model**

Denture stomatitis involves biofilm formation on a denture surface and inflammation of the adjacent oral mucosal surface (84, 85). These infections are common, occurring in up to 70% of denture wearers, and are often quite painful, even impairing the ability to eat. Biofilms are frequently polymicrobial, with Candida spp. playing a key role. Several models have been developed to explore the pathogenesis and treatment of
denture stomatitis (32, 86, 87). Early models included primarily Macaca irus monkeys with custom-fitted acrylic plates and Wistar rats fitted with prefabricated acrylic devices (88–90). The focus of these investigations was examination of the mucosal inflammatory process associated with the infected device. *Candida*-infected animals with oral devices were observed to develop mucosal lesions similar to those seen in patients with denture stomatitis (88–90). Although both models were useful for describing the host response to denture biofilms, the rat model was more suited for drug efficacy studies, primarily related to cost. In these investigations, the incorporation of either chlorhexidine or miconazole to the denture acrylic material prevented the development of mucosal lesions of palatal candidiasis (88, 89). However, the chlorhexidine product was poorly tolerated, with rats undergoing weight loss from poor dietary intake.

With the discovery of the role of biofilms in device-associated infections, there has been renewed interest in animal models to mimic denture stomatitis (32, 87, 91). Two models have been developed to replicate this clinical scenario in rats. In the first model, a Sprague-Dawley rat undergoes placement of an acrylic dental device over the hard palate, which is secured in place by orthodontic wire (32). Because the device is fitted to the individual rat, there is close approximation of the device with the oral mucosa, and this space can be inoculated with *Candida* to produce a biofilm device infection and associated mucosal inflammation over the course of 24 to 72 hours (Fig. 4). This model represents an acute infection in the setting of immunosuppression, because rats are treated with a single dose of cortisone prior to infection. In a second rat model, Wistar rats are custom fitted to palatal acrylic devices retained by orthodontic wires (87, 91). However, a portion of the device is secured by embedded magnets and is easily removable throughout the experimental course. Following inoculation of *Candida*, biofilm develops on the device surface over weeks. In addition, mucosal biofilm infection and inflammation ensue, mimicking clinical infection. Because the devices can remain in place for an extended time (8 weeks), this model offers the opportunity to longitudinally follow the course of an individual animal with a chronic infection.

Studies have only just begun to explore the antifungal treatment in the rodent denture models. As might be predicted from clinical scenarios and other infection models, the *C. albicans* biofilms on the denture surface were found to exhibit high tolerance of both fluconazole and micafungin upon either topical or systemic administration (32). The model has also been helpful for exploring the role of gene products on denture biofilm infection *in vivo*. Chen et al. described the importance of the calcineurin pathway for *C. dubliniensis* in the processes of both filamentation and biofilm formation in a rat denture biofilm (92). This suggests that calcineurin inhibitors may be a viable option for treatment of *C. dubliniensis*. Because drugs in this class exert synergistic activity with azole and echinocandin drugs, combination therapy is an attractive possibility for treatment of *C. dubliniensis* biofilm infections (92).

### Subcutaneous Implant Model

To study the activity of a novel antifungal formulation against biofilms, Zumbuehl et al. developed a murine model of subcutaneous *Candida* biofilm infection (93). Disks containing amphogel, a dextran-based hydrogel loaded with amphotericin B, were inoculated with *C. albicans* and surgically implanted in the subcutaneous flank tissue of BALB/c mice. After 3 days, *Candida* had been cleared from the surface of the disks containing amphogel. In contrast, control disks with hydrogel only were coated with *C. albicans* biofilm and host cells. The amphogel was well tolerated, eliciting only a minimal or mild inflammatory response. Because this antifungal hydrogel maintains efficacy for over 50 days, it is ideally suited for the prevention of device-associated infection.

As an alternative, simpler model for the study of *Candida* biofilm infections, Ricicova et al. fabricated a subcutaneous catheter implant model. In this model, a polyurethane catheter segment is inoculated with *Candida* and implanted under the skin of a rat (94). Over the course of 1 to 6 days, a multilayer biofilm consisting of both yeast and hyphae forms on the catheter surface. Compared to the vascular catheter model, this procedure is less invasive and requires a shorter period of anesthesia. In terms of mimicking patient infection, the model has similarities to both vascular catheter infections and wound infections. The implanted catheter material is a close mimic of the vascular catheter material used in patients. The model is avascular, so biofilm cells...

### TABLE 3 Strategies for treatment of *Candida* biofilms with demonstrated efficacy in animal models

<table>
<thead>
<tr>
<th>Lock therapies</th>
<th>Combination therapies</th>
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<tr>
<td>Liposomal amphotericin B (10 mg/ml)</td>
<td>Calcineurin inhibitor (tacrolimus) and fluconazole (49)</td>
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<tr>
<td>Amphotericin B lipid complex (5 mg/ml)</td>
<td>Hsp90 inhibitor (17-AAG) and fluconazole (49)</td>
</tr>
<tr>
<td>Caspofungin (6.67 mg/ml)</td>
<td>Diclofenac (NSAID) and caspofungin (95)</td>
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Fungal Biofilms: *In Vivo* Models for Discovery of Anti-Biofilm Drugs
are not subjected to blood flow conditions and are not exposed to the same concentrations of serum protein and blood cells. However, the devices can be treated with serum prior to implantation to partially mimic this exposure. The anatomical location of the implantation is most similar to a biofilm wound infection. The model allows for the interaction between host immune components and Candida biofilms in the subcutaneous tissue. One clear advantage is that the procedure allows for placement of multiple segments of catheter for potential comparison of several Candida strains or conditions within the same animal.

The subcutaneous implant model has also proven to be efficacious for evaluation of antibiofilm treatments. Bink et al. used this model to test the efficacy of combining a nonsteroidal anti-inflammatory drug (NSAID) and an echinocandin for treatment of C. albicans biofilms in vivo (95). NSAIDs impair prostaglandin synthesis by targeting mammalian cyclooxygenases. Agents in this class are available for the treatment of pain and inflammation. However, the activity of this drug class is not limited to mammalian systems, because they have also been shown to disrupt filamentation and biofilm formation in C. albicans, likely through inhibition of prostaglandin E2 synthesis (96, 97). To examine the impact of disrupting this pathway in vivo, rats received diclofenac treatment in the setting of subcutaneous catheter implant infection (95). In rats that had been treated with diclofenac prior to development of C. albicans biofilm infection, the antibiofilm activity of caspofungin was enhanced. It is of great interest to identify an available drug class able to potentiate the activity of echinocandins. However, the animals in the study received diclofenac prior to infection, and whether or not it would be a helpful adjuvant later in the course of infection is not known.

Urinary Catheter Model

To investigate catheter-associated urinary tract infections and candiduria, Wang et al. developed a murine model representing this clinical scenario (98). In this model, a guide wire is inserted through the urethra of a female mouse, and a catheter segment is threaded over the guide wire and into the bladder. The segment is secured by suture through the bladder wall. After 5 to 7 days, the animal is infected with C. albicans intravesicularly. Candiduria is detectable quickly after infection and persists for 28 days. A dense biofilm of adherent yeast and hyphae forms on both the luminal and extraluminal surfaces. To increase the susceptibility to Candida infection, mice lacking lysozyme M, an important effector for mucosal innate immunity, can be used. The model closely mimics patient Candida biofilm formation with regard to the use of biofilm substrate (catheter) and anatomic location (bladder). Because only a segment of catheter is in place, the flow conditions are likely less than would be observed for a patient catheter functioning to drain the bladder. The model incorporates the mammalian immune system with the option of using wild type or immunocompromised animals.

**FIGURE 4**  C. albicans biofilm infection in a rat denture model. C. albicans was inoculated between the hard palate and an acrylic device secured with orthodontic wire. After a growth period of 48 hours, the denture was harvested, fixed, and dehydrated. Oral devices were imaged by scanning electron microscopy on a JEOL JSM-6100 at 10 kV (50x and 1,000x). The biofilm is composed of both yeast and hyphae encased in an extracellular matrix. Larger host cells were observed as well. Microbiologic evaluation identified a polymicrobial infection consisting of C. albicans and various bacteria. doi:10.1128/microbiolspec.MB-0008-2014.f4
To our knowledge, this model has not yet been used for investigating the activity of antibiofilm therapies.

**Mucosal Candidiasis Models**

Biofilms have frequently been described in association with medical devices and abiotic surfaces. However, there is mounting evidence that *Candida* spp. exhibit similar characteristics when growing on mucosal surfaces (25, 26). Murine models of both oropharyngeal and vaginal candidiasis demonstrate that *Candida* produces conglomerates of yeast, hyphae, and extracellular material associated with mucosal surfaces. In an oropharyngeal candidiasis model, the biofilms appear to be complex, involving commensal bacteria, neutrophils, and keratin (25). A murine model of vaginal candidiasis shows that *C. albicans* regulators of biofilm formation on abiotic surfaces are similar to those required for the development of vaginal biofilms. Although mucosal biofilms share many characteristics with device-associated biofilms, it is not clear that they exhibit the same degree of drug resistance. Clinically, mucosal biofilms are usually responsive to antifungal therapies, including azoles (99, 100).

**Fusarium keratitis**

Outbreaks of *Fusarium* keratitis have prompted interest in models to investigate this contact lens–associated biofilm infection (101). Two murine models have been developed to investigate the pathogenesis of this process (102, 103). Sun et al. demonstrated the ability of *Fusarium oxysporum* to form a biofilm on the surface of a contact lens and for this to induce keratitis on an injured mouse cornea (102). The model has successfully been implemented for the study of the host aspects of *Fusarium* keratitis. Zhang et al. developed a similar model of murine fungal keratitis employing *Fusarium solani* (103). Although this model also involves inoculation of an injured cornea, the organisms are directly seeded and no contact lens is involved. The model utilizes fluorescently labeled fungi for visualization of the infective process.

**FUTURE DIRECTIONS**

Recognition of the importance of animal models for the discovery of antibiofilm drugs has only recently emerged. Most investigations have focused on *C. albicans* as a model pathogen, and the vascular catheter models of biofilm infection have been the most popular. It will be interesting to see how biofilms formed under the conditions of other clinically relevant niches respond to antifungal therapies. The murine urinary catheter model, the rat subcutaneous model, and rat denture models will be of great value for these investigations of *Candida* (32, 87, 94, 98). The ocular lens model should be similarly useful for identification of preventative and therapeutic compounds for fungal keratitis. The models allow for testing of antibiofilm compounds under physiologic conditions very similar to those encountered clinically. In addition to these animal models of device-associated infections, models of mucosal *Candida* biofilms will surely be helpful for the study of these common infections (25, 26).

As it is becoming increasingly clear that infections caused by diverse fungal pathogens involve biofilm communities, animal models of these infections will be beneficial for pathogenesis and drug discovery studies. Models are underway for several of the fungal pathogens. One example, discussed earlier, is *Fusarium* keratitis in the setting of contact lens biofilm infection. Murine models have been developed to study both host and pathogen aspects of this process and could be utilized to evaluate novel antibiofilm therapies in this unique clinical niche (102, 103). Also, *Aspergillus* spp., including *A. fumigatus*, have been shown to produce fungal aggregates during pulmonary infection (7). Models mimicking aspergilloma or invasive aspergillosis will be helpful for exploring the impact of antifungal treatment on this mode of growth. Although *C. albicans* has been the model pathogen for many *in vivo* biofilm investigations, the *in vivo* biofilm models can likely be adapted to biofilm infections caused by a variety of non-*albicans Candida* spp. and other yeasts, such as *Cryptococcus*. Of note, the rat vascular catheter model has been successfully used for the study of *C. parapsilosis* and *C. glabrata*, while a rat denture model has been employed for the investigation of *C. dubliensis* (92, 104).

There are many approaches to the discovery of new anti-infectives. One strategy is to screen large libraries of compounds. Using *in vitro* models, the mining of pharmaceutical and natural product libraries has identified novel compounds with antibiofilm activity (105–107). An alternative approach is to determine a mechanism leading to drug resistance and identify or develop an anti-infective that disrupts the process. For *C. albicans*, the biofilm property most closely linked to resistance is the extracellular matrix. Enzymatic degradation of key matrix components, such as extracellular DNA and β-1,3 glucan, has been shown to enhance antifungal activity, suggesting these as potential drug targets (67, 108). A therapy directed at extracellular DNA degradation has been shown to be beneficial for patients...
with cystic fibrosis. It is thought that dornase alfa (Pulmozyme), a clinically available inhaled enzymatic treatment, works by degrading extracellular DNA of bacterial biofilms (109). Regardless of the path of drug discovery, animal models will be beneficial for testing the efficacy of compounds against clinical biofilms and establishing safety.

One of the unique aspects of exploring the antibiotic activity of drugs in animal models is the opportunity to vary the mode of antifungal delivery. For example, compounds may be systemically administered, topically administered, coated on a device, or embedded in a device. Another interesting delivery method is direct administration of a gel with prolonged elution of high antifungal concentrations, such as was developed for amphotericin B (110). Systemic administration is feasible to test in all models, while direct, topical administration of a compound is easily achievable in either the denture models via topical therapy or the venous catheter models via lock therapy. The subcutaneous tissue model may be ideal for exploring the utility of embedding or coating a device with an antibiotic compound, because numerous devices can be tested in a single animal. Another potential application is the investigation of vaccine efficacy, such as the NDV-3 vaccine in clinical trials, vaccines found to be efficacious in nonbiofilm models of infections, or future vaccines designed specifically to inhibit the biofilm mode of growth (111–113).

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