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Mechanisms of *Candida* biofilm drug resistance

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Abstract

Candida commonly adheres to implanted medical devices, growing as a resilient biofilm capable of withstanding extraordinarily high antifungal concentrations. As currently available antifungals have minimal activity against biofilms, new drugs to treat these recalcitrant infections are urgently needed. Recent investigations have begun to shed light on the mechanisms behind the profound resistance associated with the biofilm mode of growth. This resistance appears to be multifactorial, involving both mechanisms similar to conventional, planktonic antifungal resistance, such as increased efflux pump activity, as well as mechanisms specific to the biofilm lifestyle. A unique biofilm property is the production of an extracellular matrix. Two components of this material, β -glucan and extracellular DNA, promote biofilm resistance to multiple antifungals. Biofilm formation also engages several stress response pathways that impair the activity of azole drugs. Resistance within a biofilm is often heterogeneous, with the development of a subpopulation of resistant persister cells. In this article we review the molecular mechanisms underlying *Candida* biofilm antifungal resistance and their relative contributions during various growth phases.

Keywords

antifungal resistance; biofilm; calcineurin; *Candida*; efflux pumps; extracellular matrix; glucan; persister cells

Antimicrobial drug resistance is an obstacle to the treatment of numerous infectious diseases [1,2]. One the most commonly recognized types of drug resistance is the ability of microorganisms to produce resilient biofilms on the surface of implanted medical devices [1–3]. When adopting this lifestyle, *Candida* proliferates as a community of adherent cells encased in an extracellular matrix. These biofilms display innate resistance to multiple drug classes and are capable of withstanding antifungal concentrations 1000-fold higher than those that inhibit nonbiofilm, planktonic cells [4–7]. Since common drug therapies do not eradicate *Candida* biofilms, removal of the infected device is almost always necessary to cure the infection [8–11]. Treatment is difficult as the medical devices are often critical for patient care and currently available antifungal therapies are virtually ineffective [7,9,12,13]. *Candida* biofilm infections, if not successfully treated, can have devastating consequences, progressing to bloodstream infections and invasive fungal infections with high risks of

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mortality. Many of the pioneering biofilm investigations, and this review, focus on *Candida albicans* [14–16]. However, it is becoming increasingly clear that numerous *Candida* spp., including *Candida glabrata*, *Candida parapsilosis*, *Candida dubliniensis* and *Candida tropicalis*, also cause recalcitrant device-associated infections.

Striking antifungal resistance is an intrinsic biofilm characteristic, and one of the many phenotypic changes that occurs upon transition to this mode of growth [8,17]. Although the resistant phenotype is most pronounced during the later phases of development, drug resistance can be detected within minutes to hours of surface adherence [8]. This resistance does not involve acquisition of genetic mutations, since biofilm cells recultured in planktonic conditions are susceptible to antifungals. *Candida* biofilm resistance to antifungal therapy appears to be multifactorial, with diverse mechanisms working in a coordinated fashion throughout the various stages of biofilm growth [18–20]. Here we will review the multiple mechanisms that contribute to the extraordinary drug resistance of *Candida* (mostly *C. albicans*) biofilms. We first include a brief overview of four drug classes commonly used to treat *Candida* infections: the azoles, the polyenes (amphotericin B), the echinocandins and 5-flucytosine.

Overview of antifungal classes with activity against *Candida* spp

Azoles

Triazole antifungal drugs inhibit growth of *C. albicans* by targeting lanosterol methylase encoded by *ERG11*. This disrupts synthesis of ergosterol, causing an increase in the 14 α -methyl sterol precursors in the cell membrane, ultimately resulting in growth arrest and susceptibility to host defenses [21–24]. Although triazoles have been the mainstay of *Candida* treatment since their development in the 1980s, acquired *Candida* drug resistance is relatively uncommon [18,25,26]. When azole resistance does emerge, it is generally seen in the setting of long-term treatment. For example, the highest rates of resistance have been reported in HIV/AIDS patients receiving treatment for oral or esophageal candidiasis pre-HAART [18,27]. Reported mechanisms of *C. albicans* azole resistance include mutations resulting in increased expression of efflux pumps (CDR1P, CDR2P and MDR1P) and mutations in the *ERG11* drug target [28–33]. Various mutations have been described, including point mutations, gene amplifications and mitotic recombination events [18,28–32,34–37]. Similar mechanisms have also been shown to play a role in azole resistance in *C. parapsilosis*, *Candida krusei*, *C. tropicalis* and *C. glabrata* [35,36,38–41]. Although acquired resistance is not common, intrinsic resistance during biofilm growth is almost universal. Cells in the biofilm environment are up to 1000-fold more azole resistant than their planktonic counterparts, making azoles an ineffective biofilm treatment option [10,20,42,43].

Polyenes

Antifungals in the polyene drug class, including the amphotericin B formulations, are thought to inhibit *Candida* through intercalation into ergosterol-containing membranes [25,44]. Although two of the less common *Candida* spp., *Candida lusitanae* and *Candida guilliermondii*, show intrinsic amphotericin B resistance, acquired resistance to amphotericin B is relatively rare. Resistance has been described in case reports from cancer patients undergoing chemotherapy and those receiving long-term prophylactic therapy [18]. The specific mechanisms of resistance to polyenes are not known, but are presumed to involve alterations in the cell membrane composition. For example, genetic strains defective in a sterol C5,6-desaturase produce little ergosterol and show clinical resistance to amphotericin B [23,45]. Sterol changes have also been linked to resistance for *C. glabrata* strains [46]. Biofilms are approximately eight-times more resistant to amphotericin B than their

planktonic counterparts, depending on the age of the biofilm [47]. Although the degree of *Candida* biofilm resistance to amphotericin B is less than that for azoles (1000-fold), the therapeutic window for amphotericin B is narrow. Doses of amphotericin B required to achieve the concentrations anticipated to significantly reduce the burden of *Candida* biofilm cells would not be safe to administer to patients. Furthermore, this therapy does not sterilize the device, allowing the biofilm to regrow following drug treatment.

Echinocandins

The echinocandins are noncompetitive inhibitors of the β -1,3 glucan synthase encoded by the *C. albicans* gene *FKS1* [48–50]; this enzyme is responsible for producing a very large percentage of cell wall carbohydrate. Depletion of cell wall β -1,3 glucan results in osmotic instability and cell lysis [51]. Resistance to echinocandins has been linked to acquired or intrinsic *FKS1* point mutations in *C. albicans* [49,50,52–56]. Similar mutations have been described for echinocandin-resistant *C. tropicalis*, *C. glabrata* and *C. krusei* strains [54,57]. Echinocandins also elicit a paradoxical effect where *Candida* is resistant to high concentrations *in vitro*. This resistance has been linked to production of a stress response, marked by increased cell wall chitin [58,59]. However, the *in vivo* significance of this resistance mechanism is unclear. Compared to planktonic cells, biofilms are approximately 2–20-fold more resistant [60,61].

5-Flucytosine

Flucytosine is brought into the cell via a cytosine permease [18]. It is then metabolized, by a cytosine deaminase in the pyrimidine salvage pathway, into a toxic version of uridine triphosphate that becomes incorporated into RNA and halts its synthesis [18,62]. Flucytosine is also converted into a metabolite that inhibits the thymidylate synthetase and thus decreases the availability of nucleotides for DNA synthesis [62]. Contemporary epidemiology demonstrates resistance rates ranging from 3 to 8% [62,63]. Non-*albicans* spp. of *Candida*, and the *C. albicans* B serotype, seem to have higher rates of intrinsic resistance [18,64]. However, the main concern with flucytosine is the rate at which *Candida* develops secondary resistance to it if used as a monotherapy. The most common causes of drug resistance are mutations in the cytosine permease gene *FCY2*, or in the cytosine deaminase gene *FCY1* [18]. *Candida* strains that are heterozygous for these mutations show partial resistance and can quickly acquire further mutations to gain full resistance upon drug exposure [18]. Flucytosine is almost always given to patients in conjunction with amphotericin B to prevent this rise of resistance [9].

Candida biofilm resistance mechanisms

The ability of *Candida* biofilms to survive extraordinarily high antifungal concentrations has been the subject of numerous investigations. Initial studies examined the impact of mechanisms known to play a role in drug resistance during planktonic growth. As described in more detail below, acquired planktonic cell resistance has been linked to increased efflux pump activity and mutations in genes encoding drug target enzymes, such as *ERG11* and *FKS1* [53,65]. In addition, alterations in the composition of both the cell membrane and the cell wall have been linked to antifungal resistance in nonbiofilm cells [58,59,65]. As it became clear that known planktonic mechanisms of resistance only accounted for a fraction of the resistance observed in biofilms, additional investigations began to focus on the role of biofilm-specific traits. These studies have examined the influence of high cell density, a slower growth rate, quorum sensing, persister cell formation, extracellular matrix production and stress responses on biofilm antifungal resistance. The role of all of these factors in biofilm resistance is reviewed below.

Role of efflux pumps

Many cases of drug resistance are linked to the augmentation of efflux pumps and the reduction of antifungal accumulation within the cell [65]. In *C. albicans*, efflux pumps have primarily been described as playing a role in azole resistance, but not in resistance to amphotericin B or the echinocandins [50,66–68]. The ATP binding cassette transporters (CDR1 and CDR2) and major facilitator transporter (MDR1) are typically expressed at low levels in the absence of antifungal exposure [69]. The finding that azole-resistant clinical isolates often show constitutive overexpression of these pumps prompted investigators to postulate that the biofilm drug resistance phenotype may be related to increased efflux pump activity [32–34,69–71].

It was demonstrated by Ramage *et al.* that transcription of both *MDR1* and *CDR1* was more abundant in 24 h *C. albicans* biofilms than planktonic cultures of the same age [72]. To investigate the role of efflux pumps during biofilm growth, the authors examined the impact of deletion of *MDR1*, *CDR1* and/or *CDR2* on drug resistance. During planktonic growth, these mutants displayed hypersensitivity to fluconazole. However, this phenotype was not observed when these same mutants were grown as biofilms for 24–48 h, suggesting that the efflux pumps do not contribute significantly to drug resistance during the mature biofilm stage [72].

Mukherjee *et al.* examined the role of efflux pumps in antifungal resistance throughout the biofilm process [73]. The researchers included early (0–11 h), intermediate (12–30 h) and mature (31–72 h) *C. albicans* biofilms with planktonic growth comparisons. Similar to the prior investigation, single, double and triple mutants of the three main efflux genes were no more susceptible to fluconazole treatment during mature biofilm growth than the parent strains; however, in the early phase (6 h), double and triple efflux pump mutants had significantly increased azole susceptibility when compared with the parent strains. Disruption of a single efflux pump had a minimal impact on biofilm resistance, even at the earliest time point. This suggests that the efflux pumps contribute to resistance during the early biofilm developmental phase, and that the pumps may function in a cooperative manner. This theory of time-specific efflux pump functionality was further supported by transcriptional analysis, showing higher expression of efflux pump genes after 12-h biofilm formation when compared with mature, 48-h biofilm formation [73].

Increased expression of efflux pumps during biofilm growth has been confirmed in a rat venous catheter model when compared with planktonic cultures of the same age, including *CDR2* at 12 h and *MDR1* at 12 and 24 h [74]. Investigations of *C. glabrata* and *C. tropicalis* biofilms have also shown upregulation of efflux pumps [20,41]. This is collective evidence that *Candida* efflux pumps likely contribute to drug resistance during the early phase of biofilm growth, while their role in resistance in mature biofilms appears to be minimal at most. It is possible that the efflux pump activity may be overshadowed by other resistance mechanisms, or that the pumps may start acting more on non-drug substrates, such as environmental toxins, in these later hours.

Influence of sterol synthesis

The observation that *Candida* mutants with impaired ergosterol synthesis demonstrate enhanced resistance to azoles and amphotericin B led investigators to question if *Candida* biofilms may employ a similar mechanism to withstand high antifungal concentrations [73]. Mukherjee *et al.* examined the sterol composition in biofilms during the various phases of biofilm formation [73]. Compared to planktonic cells, the cell membranes of biofilm cells contain a significantly lower concentration of ergosterol, especially during the later phases of biofilm growth [73]. Cells in mature biofilms contain approximately half the ergosterol

content of those in the early phase of growth. This finding suggests that the mature biofilms rely less on ergosterol for maintaining membrane fluidity, potentially limiting the efficacy of the ergosterol-targeting drugs, such as azoles and amphotericin B.

Several studies examining the various phases of biofilm formation have shown alterations in the transcriptional profile of sterol pathway genes [8,74]. Microarray analysis demonstrated increased transcription of both *ERG25* and *ERG11* during *in vitro* *C. albicans* biofilm growth when compared with planktonic cells of the same age. *ERG11* encodes the azole drug target and *ERG25* encodes a putative C4 methyl sterol oxidase, thought to play a role in C4-demethylation of ergosterol biosynthesis intermediates [75]. This enzyme has been proposed to promote increased conversion of lanosterol to nonergosterol intermediates, including eburicol and 14-methyl fecosterol, at the expense of the conversion to ergosterol [74]. Interestingly, transcriptional analysis of a rat venous catheter biofilm also found increased transcription of *ERG25*, but not *ERG11* [74]. It is thought that altered ergosterol synthesis likely contributes to *Candida* biofilm resistance. However, studies have primarily involved correlative findings and a definitive link has not been established.

Role of cell growth rate

Another hypothesis for antifungal resistance is that cells that are deeper in a biofilm grow more slowly owing to a lack of nutrients, and are subsequently more resistant to antifungal drugs that rely on cell growth for their effects. This has been shown to be the case for certain bacterial biofilms [76,77]. By controlling nutrients in a perfused biofilm fermentor, Baillie and Douglas were able to compare the antifungal susceptibility of biofilms growing at various rates [78,79]. Over a wide range of growth rates, biofilm-associated cells exhibited similar levels of resistance to amphotericin B, suggesting that growth rate does not play a significant role in biofilm antifungal resistance.

Impact of cell density & quorum sensing

Another biofilm-specific trait suspected to influence drug susceptibility is the relatively high concentration of fungal cells in a biofilm compared with many planktonic conditions [80,81]. To examine the role of high cell density on antifungal resistance, Perumal and Chaffin compared the susceptibility of planktonic yeast cultures with intact and disrupted biofilms [80]. Cells from each of these conditions showed greater resistance to azole drugs at a high cell density than at a low cell density (an inoculum effect). This finding suggests that the high cell density of biofilms influences antifungal resistance; however, this is not an attribute that applies only to biofilm cells.

Quorum sensing and cell density are closely linked in *Candida* biofilms. Two key quorum sensing molecules in *C. albicans*, tyrosol and farnesol, exert opposing activities [82–84]. Farnesol has been shown to inhibit both biofilm formation and the development of hyphae [84,85]. This effect has been observed for *C. albicans* biofilms as well as *C. dubliniensis* [86,87]. Microarray analysis of farnesol-treated biofilms first suggested a link between quorum sensing and drug resistance [88]. Biofilms exposed to farnesol were found to have decreased transcription of *PDR16*, an ATP binding cassette transporter involved in planktonic azole resistance, and increased transcription of *FCR1*, a zinc-cluster transcription factor known to negatively regulate drug resistance. However, the finding that farnesol is present at high concentrations during the mature biofilm phases, the height of biofilm resistance, questions the relevance of differential expression of these genes in biofilm resistance. In addition, a *CHK1* mutant defective in farnesol response does not demonstrate increased fluconazole susceptibility in high density conditions [80].

Contribution of biofilm extracellular matrix

A key feature of mature biofilms is the production of extracellular matrix, a polymeric material that promotes adherence and protects biofilm cells from environmental insults. This material is also thought to aid in the retention of nutrients, water and enzymes [85,89]. Investigations have questioned if the biofilm matrix material may also impair drug delivery, either via steric hindrance or by actively binding or sequestering antifungals. The Douglas group performed the first studies in this realm. Through altering growth conditions, they were able to produce biofilms with different quantities of extracellular matrix. Compared with statically grown biofilms, those that were grown in a shaking incubator produced visibly more matrix by electron microscopy. Despite the difference in matrix abundance, the biofilms were equally resistant to amphotericin B, fluconazole and flucytosine [90]. It wasn't until they examined biofilms grown under continuous flow, a condition promoting the highest matrix quantity and more closely resembling *in vivo* conditions, that they were able to link *Candida* biofilm resistance to the production of an extracellular matrix [91]. A correlation between the degree of extracellular matrix production and antifungal resistance was also found for *C. tropicalis* biofilms [91].

Nett *et al.* explored the potential action of biofilm matrix on antifungal resistance by measuring the influence of purified matrix material added to planktonic cells [92]. The observation that the addition of biofilm matrix to nonbiofilm cells mimicked the biofilm drug-resistant phenotype prompted the theory that matrix may be binding or sequestering drugs, preventing them from reaching their intracellular target. Indeed, using radiolabeled fluconazole, biofilm matrix was found to sequester azole drugs. This resistance appears to correlate with production of a matrix carbohydrate, β -1,3 glucan [92–94]. Vedyappan *et al.* found that biofilm matrix also interacts with amphotericin B, and implicated this interaction in biofilm drug resistance [95]. Additional studies support a similar biofilm resistance mechanism for a variety of antifungals, including flucytosine, and the echinocandin drug class [96]. Bio-films formed by other *Candida* spp., including *C. glabrata*, *C. parapsilosis* and *C. tropicalis*, also display a matrix-antifungal sequestration mechanism of drug resistance [97]. Compared with other available antifungals, the echinocandin drug class appears to have more antibiofilm activity. One explanation for this is that these drugs inhibit β -1,3 glucan synthesis, possibly impairing production of the critical matrix component [98].

Several investigations have searched for the genetic basis underlying matrix antifungal sequestration. The first of these linked the major *C. albicans* glucan synthase, FKS1, to production of extracellular matrix glucan and to the biofilm drug-resistant phenotype, both *in vitro* and in an animal model [93]. This pathway appears to be under the control of regulator SMI1 through the transcription factor RLM1 [99]. The Mitchell group identified a link between *ZAP1* and the manufacture of *C. albicans* biofilm matrix. As a negative regulator of matrix glucan production, mutants deficient in *ZAP1* accumulate high quantities of extracellular biofilm matrix [100]. Target genes regulated by *ZAP1* encode two glucoamylases (GCA1 and GCA2) and three alcohol dehydrogenases (ADH5, CSH1 and LFD6). With positive roles in matrix production, these enzymes are hypothesized to participate in the production of soluble β -1,3 glucan, perhaps through production of molecules involved in a quorum sensing process.

An investigation by Taff *et al.* examined the delivery process of glucan-containing components to the extracellular matrix. This pathway appears to be modulated by several gene products, including two predicted glucan transferases (BGL2 and PHR1) and an exoglucanase (XOG1) [94]. These enzymes act in a complementary fashion to modify and distribute matrix downstream of the primary β -1,3 glucan synthase FKS1. Disruption of this matrix delivery system impairs biofilm matrix β -1,3 glucan production, biofilm adherence and resistance to azole drugs. Together, these studies show that the transition to a biofilm

lifestyle involves a coordinated cellular response to produce and distribute extracellular matrix.

Another recently identified matrix component impacting drug resistance is extracellular DNA [101,102]. This is based on the finding that treatment of *C. albicans* biofilms with DNase augments the activities of echinocandins and polyenes [102]. Surprisingly, DNase treatment has no impact on the activity on fluconazole against *Candida* biofilms. The potentiation of drug activity upon DNase treatment of biofilms is not unique to *Candida*. In bacterial biofilms, this effect is related to exchange of genetic material [103,104]; however, a similar role in *Candida* has not been described [3,105,106]. It is unclear how matrix extracellular DNA contributes to *Candida* biofilm resistance and if this pathway is distinct from the sequestration mechanism of resistance.

Presence of persister cells

An intriguing development in understanding *Candida* biofilm resistance is the discovery of persister cells, a subset of cells that lie deep in a biofilm and exhibit tolerance to multiple drug classes, including amphotericin B, azoles and chlorhexidine. Persister cell formation is characteristic of both *Candida* and bacterial biofilms [107,108]. These dormant variants can serve as the inoculum for a new biofilm containing the same percentage of persister cells [107]. While the mechanism of *C. albicans* persister cell transition remains unclear, it is understood that they form only upon adherence to a surface. Transcriptional analysis of these cells shows differential regulation of genes involved in both the ergosterol (*ERG1* and *ERG25*) and β -1,6 glucan (*SKN1* and *KRE1*) pathways [109]. These results suggest the possibility that the transition to a persister cell involves changes in both the cell membrane and the cell wall. Additional studies have shown that the ability to form persister cells is both species- and strain-specific. For example, *C. albicans* and *C. krusei* spp. biofilms frequently exhibit a persister cell subpopulation, while it is suggested *C. glabrata* spp. do not [110,111].

Influence of biofilm stress responses

Candida adherence and biofilm growth involves the activation of several stress responses that promote drug resistance. The first investigation in this realm examined the role of the MAPK Mkc1p, a key component of the signal transduction pathway triggered by cell wall stress [112]. *C. albicans* mutants with disruption of *MKC1* were found to be defective in hyphal formation, biofilm development and biofilm-associated resistance to fluconazole. Subsequent investigations have examined the influence of additional cell stress regulatory pathways on biofilm resistance. Uppuluri *et al.* identified a role for calcineurin, a Ca^{2+} calmodulin-activated serine/threonine-specific protein phosphatase important for homeostasis, morphogenesis and virulence [113]. Inhibition of this pathway through either genetic disruption (*CNB1* or *CRZ1*) or pharmacologic therapy (FK506) improved the activity of fluconazole against *C. albicans* biofilms. Another stress response pathway contributing to *Candida* biofilm resistance involves HSP90, a heat shock protein responsible for stabilization of a variety of host proteins [114]. In planktonic conditions, HSP90 associates with both calcineurin and MCK1. These chaperone clients have not been demonstrated during biofilm growth [114]. Therefore, there appears to be a distinct stress pathway promoting *C. albicans* biofilm resistance that involves HSP90. The finding that disruption of this pathway also impairs matrix glucan production suggests the possibility that HSP90 may act as a regulator of the matrix sequestration pathway [114].

Conclusion

Upon transitioning to the biofilm lifestyle, *Candida* becomes extraordinarily resistant to anti-infectives and host defenses. The processes that *Candida* spp. employ to withstand conventional antifungals is multifactorial, and vary with the phases of biofilm formation. In addition to mechanisms accountable for planktonic resistance, biofilms also utilize pathways specific to the biofilm mode of growth (Figure 1).

During the early phase of biofilm development, efflux pumps account for a portion of the observed drug resistance [73]. This mechanism of resistance involves a transient increase in pump activity during the stages of adherence and biofilm formation. This is distinct from acquired resistance, which involves a genetic mutation that results in increased pump activity. However, as the biofilm matures, the role of the efflux pumps is minimized and biofilm-specific mechanisms play a much larger role. Mature biofilms produce an extracellular matrix containing β -1,3 glucan that participates in sequestration of antifungals, including fluconazole, amphotericin B and flucytosine. The extracellular DNA of the matrix also promotes resistance to both amphotericin B and the echinocandins [102]. The activation of stress-induced pathways, including the calcineurin, HSP90 and mitogen-activated pathways, contributes to *Candida* biofilm resistance by a variety of mechanisms, including control of matrix production. Mature biofilms often develop persister cells, phenotypic variants that are resistant to antifungals and provide an inoculum for regrowth after drug exposure [107]. In addition, there are other biofilm-specific characteristics, such as a decrease in cell membrane ergosterol content that would be expected to participate in resistance to ergosterol-targeting antifungals. However, rigorous investigation to establish this link has not been undertaken.

Future perspective

There are many overlapping and redundant mechanisms that allow biofilms to exhibit drastic drug resistance, often resulting in treatment failure for patients. By understanding which mechanisms are dominant under specific conditions, we can hopefully optimize the current therapies available and identify novel approaches for treating these resilient infections. New drugs designed to target biofilm-specific mechanisms of resistance may be able to overcome the limitations of current therapeutics.

Production of an extracellular matrix is one of the key resistance mechanisms for *Candida* biofilms [91–94,97]. Both carbohydrate and extracellular DNA components have been linked to multidrug resistance [92,94,97,101,102]. It is quite possible that this complex polymeric substance may contain other compounds impacting antifungal resistance as well. Biochemical compositional analysis, including structural analysis of the major matrix components, may be helpful for the development of novel therapies based upon component synthesis inhibition [16,90,91]. Furthermore, studying matrix composition under different conditions might account for variations in its functionality. For example, matrix varies among stationary, shaking and continuous flow conditions [90,91]. In addition, *in vivo* biofilms are exposed to numerous host factors that may be incorporated into the matrix and promote a greater level of drug resistance than that observed for *in vitro* biofilms [85,91]. Future investigations addressing these questions may provide insight into the importance of specific matrix components for the structural integrity and antifungal resistance phenotype of *Candida* biofilms.

The recent discovery of persister cells in *Candida* biofilms has sparked great curiosity about their role in biofilm drug tolerance. The major gap in information about these cells lies in their mechanism of formation. The production of reactive oxygen species within the biofilm

may be connected. Investigations of azole-treated *C. albicans* biofilms found that the persister cell population produced increased reactive oxygen species compared with the rest of the biofilm [115,116]. Strains that lack superoxide dismutase production, due to disruptions of genes *SOD4* and *SOD5*, formed significantly fewer tolerant persister cells, indicating that this process may be linked to the ability of biofilms to persist through drug treatment [115]. However, those mutated cells may have additional deficits that prevent the formation of persister cells, unrelated to superoxide dismutase production. Future studies addressing which environmental or host conditions promote formation of persister cells may be of value. For example, prolonged antimicrobial treatment of mucosal infections has clinically shown to select for *Candida* strains with relatively larger populations of persister cells [111]. Understanding the regulation of persister cell formation may identify novel therapeutic targets, and agents preventing this resistant subpopulation would be ideal for combination therapy with already available antifungals.

Biofilm quorum sensing is another intriguing target for the design of novel, biofilm-specific therapeutics. It is likely that *Candida* utilizes other quorum-sensing molecules in addition to farnesol and tyrosol. For example, two aromatic alcohols, tryptophol and phenylethanol, secreted by *C. albicans* under certain conditions could very well be involved in quorum sensing, but are not yet confirmed players [117]. In addition, it is possible that signaling among species in mixed biofilm infections could involve yet more additional molecules. This may be particularly relevant for oral biofilms. Defining these pathways, in addition to understanding the specific drug resistance responses to the various quorum-sensing molecules, may reveal novel targets for drug development [88,118,119]. Promising results have been published on inhibiting bacterial biofilms by targeting their quorum-sensing mechanisms [120]. In *C. albicans*, exploitation of farnesol has been proposed for this purpose [72]. However, this molecule has not been definitively linked to drug resistance and has the potential to promote dissemination of infection to distant sites [85,121,122]. Future studies that examine the possibility of alternative quorum-sensing molecules or conclusively determine the role of farnesol in cell dispersion may lead to more ideal drug targets.

Data from *in vitro* experiments and animal models suggest that the echinocandins are perhaps the best current antifungal option for use on mature biofilms [98,123,124]. Liposomal amphotericin B has also been shown to have modest activity against *Candida* [98,124,125]. Despite these *in vitro* and animal activities, none of the currently available antifungal therapies have been shown to be effective in patients with *Candida* device-associated biofilm infections. In fact, the current treatment guidelines recommend device removal for treatment of *Candida* biofilm infections, as these often recur if treated with antifungals alone [9–11,126]. Similarly to the β -lactamase inhibitors used to overcome β -lactam bacterial resistance, drugs active against the biofilm-specific drug resistance mechanisms discussed above may have the potential to eradicate biofilms in combination with other antifungals. Many laboratories have begun to explore this possibility [102,127–134]. The finding that stress responses are involved in *Candida* biofilm resistance raises the possibility of inhibiting these pathways to potentiate the effects of conventional antifungals. For example, FK506, a calcineurin inhibitor, is currently available for patient use as an immunosuppressant. In an animal model, this was shown to have synergy with fluconazole when administered as lock therapy for treatment of *C. albicans* venous catheter infection [113]. Other drugs targeting stress responses, or other biofilm-specific mechanisms of resistance, may also improve the activity of conventional antifungals, eradicating these resistant infections.

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Executive summary

Background

- *Candida* spp. frequently form drug-resistant, difficult-to-treat biofilms on the surface of medical devices, such as vascular and urinary catheters.
- Biofilm formation involves a phenotypic transition associated with up to a 1000-fold increase in resistance to antifungal therapy.
- Available antifungal therapies are not effective against *Candida* biofilms. Device removal is often required to cure these infections.

Candida biofilm resistance properties

- *Candida* exhibits intrinsic, reversible, multidrug resistance when growing as a biofilm.
- Biofilms frequently develop a subset of extremely drug tolerant 'persister cells' that can serve as an inoculum for new biofilms following drug treatment.

Candida biofilm resistance mechanisms

- During the early phase of biofilm growth, increased efflux activity promotes resistance by reducing the intracellular accumulation of azole drugs.
- Extracellular matrix, a defining characteristic of *Candida* biofilms, sequesters antifungal drugs, including azoles, amphotericin B and flucytosine. The sequestration prevents the drugs from reaching their cellular targets. This mechanism has been linked to production and delivery of β -1,3 glucan to the matrix.
- *Candida albicans* biofilm matrix contains extracellular DNA, which promotes resistance to amphotericin B and the echinocandins.
- Several stress-induced pathways are activated during biofilm growth and contribute to azole resistance. These include the MAPK pathway, the calcineurin pathway and the HSP90 pathway.
- As the biofilm matures, the observed alterations in the cell membrane sterol composition are hypothesized to impair the activity of some antifungals.

Conclusion

- Multiple mechanisms contribute to the intrinsic antifungal resistance of *Candida* biofilms.
- Factors accounting for biofilm resistance vary among growth phases.
- The high degree of resistance observed for *Candida* biofilms suggests there may be undiscovered mechanisms that also play a role in biofilm drug resistance.
- New therapeutic agents targeting biofilm-specific resistance mechanism are desperately needed.

Figure 1. *Candida albicans* biofilm resistance mechanisms
Resistance mechanisms at the (A) community and (B) cellular level.