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Candida albicans Biofilms, Heterogeneity and Antifungal Drug Tolerance

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Abstract: *Candida albicans* is a pathogenic member of the human oral and gastrointestinal microbiota. Biofilms of *C. albicans* form on indwelling devices, such as catheters and heart valves, and recent evidence suggests that biofilms also form on the mucosal surfaces of the mouth and vagina. Biofilm infections of prosthetic devices are untreatable by antifungals and infections of the mucosa are frequently difficult to treat and recurrent. The recent characterizations of the attachment mediated tolerance of persister cells to amphotericin B and calcineurin-Hsp90 based tolerance to azole antifungals have helped to shed light on the recalcitrant nature of *C. albicans* infections. In this review, the refractory nature of *Candida* infections to antifungals will be examined through the context of biofilms, heterogeneity and drug tolerance. Understanding the mechanisms of biofilm drug tolerance and phenotypic heterogeneity may lead to the development of the first antifungal drugs capable of eradicating infection, salvaging medical devices, and preventing relapse.

Keywords: Persister, antifungal resistance, biofilm formation.

BIOFILM RELATED REFRACTORY CANDIDIASIS

Candida biofilms have been reported on most indwelling medical devices and frequently occur on blood and urinary catheters, dentures, voice prosthetics and artificial joints [1]. These infections are often untreatable with antimicrobial therapy and require device removal [2]. Failure to remove an infected medical device in a timely manner can result in life threatening disseminated candidiasis [3,4]. Since it is not always possible or convenient to remove medical devices, attempts to salvage infected prosthetics have been made, but have largely failed [5]. These risks were highlighted in a study of patients with fungal infected ventricular assist devices, which reported a 91% mortality rate [6]. New evidence suggests that *in vivo* biofilms also form on the mucosal surfaces of the mouth and vagina in mice [7,8]. In each case, populations of *Candida* were observed microscopically that met the criteria of biofilm - cells were attached to the mucosal surface and consisted of a mixture of closely associated yeast and hyphal morphologies that were surrounded by an extracellular matrix [7,8]. These studies raise the interesting possibility that the refractory nature of mucosal *Candida* infections could be in a large part due to the presence of biofilms.

It is estimated that 75% of women will develop vaginitis caused by *C. albicans* at least once [9]. Infection is most likely caused by alterations in the vaginal microflora due to hormonal fluctuations or antibacterial therapy [10], and most cases are easily treatable with azoles [2]. However, according to the U.S. Centers for Disease Control and Prevention, 10-20% of cases either resist therapy all together or relapse [9]. A 6 month prophylactic fluconazole treatment for recurrent vaginitis decreased relapse two-fold, but did not provide a long-term cure [11,12]. Fluconazole is the drug of choice for most clinicians since it is effective, more convenient and better tolerated compared to topical therapies. However, in patients with HIV, oropharyngeal, esophageal and vaginal candidiasis, infections can be recurrent and debilitating [13]. Approximately 60% of HIV patients experience a recurrence within 6 months of the initial episode [14]. Patients that do not respond to fluconazole may be given amphotericin B, caspofungin or voriconazole, but these drugs are limited by IV administration, safety profiles and drug-drug interactions [13,15]. Refractory oral candidiasis is currently reported in 4-5% of HIV infected individuals [13]. Prophylactic therapy for recurrent mucosal candidiasis in not recommended due to resistance development that could complicate treatment of systemic disease [2]. Thus, there is an urgent need to develop additional therapeutic options for patients whose infections are recurrent or refractory, and may be biofilm associated.

CANDIDA BIOFILM FORMATION

C. albicans biofilms form when single cells attach to a surface and grow into microcolonies, which then merge and produce a complex 3-D structure that is held together by hyphae and an exopolymer matrix [16]. The biofilm is heterogeneous containing a mixture of yeast, hyphae and pseudohyphae forms [17]. Yeast attach to a surface through physical properties of the cell, such as hydrophobic and electrostatic interactions, and with the aid of adhesin proteins [18]. The agglutinin-like sequence (ALS) adhesins are glycophosphatidylinositol anchored cell wall proteins that mediate cellular attachment to other cells and surfaces. Several genes encoding adhesins have been identified by sequence conservation and experimental evidence including, Als1-7, Als9-12, Hwp1 and Eap1 [18]. Many adhesins are

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redundant in function [19], yet differentially regulated under a variety of environmental and stress conditions [20,21]. In addition, adhesin genes are subject to both genotypic and phenotypic plasticity in the form of tandem repeat recombination and epigenetic histone code modification [18]. Adhesins are responsible for the ability of *Candida* to attach to a wide range of cell types and surfaces. Once attached, cells grow, divide and form microcolonies which merge, while adhesins stabilize the entire structure through cell-to-cell interactions [22]. In wild type biofilms, a layer of predominantly hyphae forms on top of a basal layer of yeast, and the biofilm can be over 500 µm thick [23,24]. Water channels and extracellular matrix pervade the entire structure.

A number of genes have been identified that are necessary for the wild type biofilm development or maintenance described above. For example, Nup85, Mds3, Kem1, and Suv3 were identified as genes required for wild type biofilm formation since homozygous deletion caused a less opaque appearance on silicone and more turbid growth in medium in a biofilm formation assay [25]. Subsequent analysis revealed all of these strains were defective in hyphae formation [25,26]. A similar screen of transcription factor mutants revealed that *bcr1*Δ produced a biofilm of greatly reduced biomass and thickness, despite the fact this strain was able to produce hyphae [27]. Bcr1 regulates expression of adhesins, such as Als1, Als3, and Hwp1 [28]. In contrast, Ywp1 is a negative regulator of adhesion, which may aid in biofilm cell dispersion [29]. *ywp1*Δ caused increased attachment to surfaces and other cells, however cells overexpressing Ywp1 do still attach to surfaces, although at a reduced level compared to wild type [29]. Nrg1, a negative regulator of hyphae formation, also regulates biofilm dispersion [30] and the transcription factor, Zap1, is a negative regulator of biofilm matrix production [31]. These data suggest that any mutation that causes a defect in attachment, filamentation or growth [32] will cause a biofilm defect. Typically, biofilm defective mutants still attach to a surface and form a congregation of cells albeit with reduced biomass. These attached cells may be less adherent and more easily washed away compared to wild type biofilms.

Biofilms have many unique phenotypic properties and gene expression patterns of biofilm cells differ dramatically compared to planktonic cells [33-35]. Extracellular matrix, adhesin production and drug efflux transporters are all upregulated in biofilms [22,36,37]. In addition, microarray analysis of biofilms points to wholesale changes in protein synthesis, metabolism (amino acid and nucleotide), transcription and cellular organization [33]. For example, when gene expression patterns were followed 30 minutes to 6 hours after attachment, many genes involving sulfur assimilation and metabolism were upregulated compared to age matched planktonic controls [34]. Another study, comparing gene expression patterns of 6, 12 and 48-hour biofilms to planktonic cells and each other [35] also noted changes in metabolism and transport, but few differences were detected between early and late stage biofilms [35]. Taken together, these studies reveal that a common subset of genes is expressed in biofilms, different from those expressed during planktonic growth. These changes begin almost immediately after attachment to a surface. For the purposes of this review, a biofilm will be defined as a surface attached population of cells with properties distinct from planktonic cells.

The drug resistant phenotype of biofilms supports the aforementioned broad definition of a biofilm. Upregulation of drug efflux pumps is detectable by RT-PCR after only 15 minutes of surface attachment and increased resistance to the antifungal fluconazole was detectable within 2 hours [38]. In addition, higher order biofilm structure does not appear to be necessary for drug resistance. Analysis of morphological mutants, stuck in either yeast or hyphal form and unable to transition between the two, revealed that both morphologies were able to attach to a surface and aggregate [23]. Importantly, each population displays a drug resistant phenotype compared to planktonic cells [23]. Similarly, *efg1Δ* and *cph1Δ/efgΔ* strains, which are defective in filamentation, produce monolayer biofilms that are resistant to both amphotericin B and fluconazole [26]. Surface attachment appears to be a key factor in mediating biofilm drug resistance. Interestingly, the attachment mediated resistance of cells to fluconazole is at least partially dependent on Mkc1 [39]. Mkc1 is a contact dependent kinase required for invasive growth into agar. While a *mkc1*Δ mutant was able to form a defective biofilm, the attached cells were remarkably susceptible to fluconazole as measured by 2,3-bis(2-methoxy-4-nitro-5-sulfophenly)-5-[(phenylamino) carbonyl]-2H-tetrazolium hydroxide (XTT) reduction, an indicator of metabolic activity. When Mkc1 was complemented back into the deletion strain, fluconazole resistance was restored to the wild type level [39].

The inherent drug resistance of attached biofilm cells compared to planktonic cells has been reported for almost every antifungal and even some biocides [40]. Resistance is attributed to a variety of factors, and supporting or contradictory evidence may only apply to specific drugs or drug classes.

ANTIFUNGAL DRUGS

The first systemic antifungals were the polyenes, such as nystatin and amphotericin B. These drugs were discovered in the early 1950's by screening large collections of *Streptomycetes* cultures for antifungal activity [41]. Polyenes act by binding ergosterol, a fungal specific component of the cell membrane analogous to mammalian cholesterol [42]. Amphotericin B creates pores in the cell membrane by forming aggregates with ergosterol. Pore formation was initially inferred by measuring extracellular ion (K^+) released from cells after amphotericin B treatment [43,44]. Subsequent studies used linear dichroism-FTIR and molecular modeling within membranes to suggest amphotericin B interacts with ergosterol and specifically disrupts membrane polar head groups [45,46]. Polyenes are lethal to *Candida*, resistance development is extremely low, and despite the nephrotoxicities associated with them, these drugs are still in clinical use. Amphotericin B has excellent *in vitro* activity against biofilms [47], but is not completely effective [48-51].

The next major class of antifungals is the azoles. *In vitro* antifungal efficacy of benzimidazole was first reported in the 1940's [52]. Azoles inhibit 14-alpha lanosterol demethylase, the ERG11 gene product in *Candida*, leading to ergosterol

depletion and growth arrest [53]. First generation azoles with an imidazole backbone, such as miconazole and clotrimazole, are still considered effective treatments for topical infections. However, systemic imidazole use was replaced with the invention of the triazoles in the 1990's. Triazoles, including fluconazole and voriconazole, were favored over imidazoles due to increased water solubility, broader spectrum of activity, and lower toxicity [52]. Newer triazoles include posaconazole, ravuconazole, isavuconazole and albaconazole. The azoles are not lethal to *Candida*, although strain specific lethality has been reported [54] and may be due to the accumulation of the toxic ergosterol precursor 14-α-methyl 3,6 diol [55] or additional mechanisms of action at higher concentrations [56]. The azoles are ineffective against biofilms.

The first enchinocandin, caspofungin, was licensed in 2001 and marked the arrival of a new class of antifungals [57]. Other drugs in this class include micafungin and anidulafungin. Enchinocandins inhibit glucan synthesis, presumably by blocking the enzyme beta-1,3-glucan synthase, causing defects in the fungal cell wall [58]. Caspofungin is lethal to *Candida*, has an excellent safety profile and is reported to kill biofilms *in vitro* [59]. However, the killing effect of enchinocandins against biofilms is incomplete [52,60-64]. The paradoxical effect is well documented for caspofungin [65,66], and has been implicated in the survival of biofilms to this drug [67]. The killing of *C. albicans* by enchinocandins is unclear, since yeast can survive as spheroplasts that lack cell walls. Killing by enchinocandins may be due to lysis caused by a combination of cell wall and osmotic stresses, and may be largely dependent on media and growth conditions. Clinically, enchinocandins are insufficient to treat biofilm infected prosthetics. Some currently used antifungal agents, their drug classifications and mechanisms of action are listed in Table **1**. In order to effectively treat biofilm infected prosthetics additional potent and cidal antifungal drugs are needed.

BIOFILM DRUG RESISTANCE

Biofilms exhibit increased drug resistance compared to genetically identical planktonic cells. Several factors appear to be involved in this phenomenon. The exopolymer matrix of biofilms restricts penetration of immune system components [68,69]. Since antimicrobials act along with the immune system to eradicate infections *in vivo*, the biofilm exopolymer matrix is an important component of recalcitrance. In *Candida*, the matrix consists of carbohydrate, protein, hexosamine, phosphorus and uronic acid [70]. Any of these components have the potential to bind antifungals, restrict access to cellular targets, and confer clinically relevant resistance.

It is likely that direct binding and sequestration of antifungals to the exopolymer matrix has an effect on resistance, but different studies have provided conflicting data. A disk filter technique showed the exopolymer matrix did not appreciably hinder penetration of fluconazole or amphotericin B through the biofilm [71,72]. In addition, biofilms that produced more matrix due to turbulence exhibited similar susceptibility to amphotericin B and fluconazole compared to static biofilms with less matrix [71]. However, a similar experiment comparing continuous flow growth conditions with more matrix to static biofilms reported a decreased susceptibility to amphotericin B, although not fluconazole [37]. (1→3)-β-D-glucan, which is more prevalent in biofilms compared to planktonic cells, has been shown to directly bind fluconazole and contribute to resistance [73,74]. In addition, biofilm spent medium, but not supernatant derived from planktonic culture, was reported to bind fluconazole [75]. The binding of fluconazole to (1→3)-β-D-glucan of the biofilm extracellular matrix probably reduces the freely soluble concentration of drug available to enter the cells. The matrix may bind to other antifungals in addition to fluconazole and contribute of biofilm drug resistance, although matrix binding by the polyenes or enchinocandins has not been examined systematically.

Quiescence or slow growth is a factor in bacterial drug resistance. However, studies utilizing XTT and 2-chloro -4- (2,3-dihydro-3-methyl- (benzo-1,3-thiazol-2-yl)-methylidene)-1- phenylquinolinium iodide (FUN-1) demonstrated that the majority of cells in *C. albicans* biofilms are metabolically active [47,63,76]. In fact, the increased metabolic activity of biofilms correlated with increased resistance to fluconazole and amphotericin B [16]. Biofilm resistance to amphotericin B was also determined to be largely independent of growth rate in a perfused biofilm fermentor setup [77].

Resistance in planktonic populations of cells may point to mechanisms involved in biofilm drug resistance. For example, genes encoding multidrug resistance (MDR) transporters *MDR1*, *CDR1* and *CDR2* are important in fluconazole resistant clinical isolates [53] and are upregulated transiently in heteroresistant colonies [78,79]. Transcriptional profiles have also shown these transporters are upregulated upon attachment of *C. albicans* cells to a surface, accounting for the resistance of young biofilms to azole antifungals [80]. However, the high level of drug resistance of mature biofilms (≥48 hour) was not affected by deletion of any of these genes, including a *mdr1*Δ *cdr1*Δ

Table 1. The Drug Classification, Mechanism of Action and Examples of Some Currently Used Antifungals

Class	Mechanism of Action	Examples
polyene	binds ergosterol; creates pores in the cell membrane	amphotericin B, nystatin
azole	inhibition of 14-alpha lanosteroldemethylase; accumulation of 14-alpha-methyl 3,6 diol	fluconazole, voriconazole, itraconazole, posaconazole
echinocandin	inhibition of cell wall glucan synthesis by beta-1,3 glucan synthase	caspofungin, micafungin, anidulafungin

*cdr2*Δ triple mutant [36,39,80]. Apparently drug efflux is not a factor in resistance of mature biofilms to the azoles and, in addition, the echinocandins and the polyenes are not substrates of any known drug transporter.

Drug target mutation can cause resistance, but is unlikely to occur universally in biofilms. Genetic target mutations cause stable resistance, yet biofilm drug resistance is largely transient [36,81]. Transient downregulation of a drug target may cause resistance and many antifungals including the polyenes and the azoles, target ergosterol, an essential component of the fungal cell membrane. Indeed, a profile of membrane sterol composition revealed decreased ergosterol content in mature biofilms [39,80]. Diminished ergosterol biosynthetic gene expression [33] has also been reported in biofilms. However, ergosterol depletion is unlikely to be involved in resistance to the echinocandins that inhibit the synthesis of cell wall (1→3)-β-D-glucan[58], or chlorhexidine [47], a membrane-active antiseptic.

BIOFILM HETEROGENEITY AND DRUG TOLERANCE

Heterogeneity may cause drug tolerant subpopulations of cells to exist within a biofilm [49,51,82-86]. The appreciation of heterogeneity, or different responses and phenotypes within a genetically identical population of cells, represents a recent paradigm shift in the field of biofilms and microbiology in general [87]. This small proportion of cells could be important for the overall success of drug therapy, since biofilms are protected from immune components by the extracellular matrix *in vivo*. Unique subpopulations of cells within a biofilm have been documented in a variety of different circumstances. A biofilm was found to express stationary phase specific genes SNZ1 and SNO1 exclusively in a small population of yeast cells closely attached to the substrate [82]. It is unknown whether these stationary subpopulations of cells have increased resistance to antifungals. Increased resistance to amphotericin B was detected in a subpopulation of biofilm cells that remained closely associated with the surface after most cells were washed away by increased flow rate of a perfusion chamber [51]. A 10 fold decrease in susceptibility to amphotericin B was measured for these cells and resistance correlated with the differential expression of ergosterol and glucan synthesis genes ERG1, ERG25, SKN1, and KRE1 [51]. Cells at the base of a biofilm were similarly reported to have increased resistance to chlorhexidine [84]. A separate subpopulation of live cells was detected within biofilms after exposure to a lethal concentration of caspofungin [47] and these cells retained metabolic activity in the presence of caspofungin as reported by FUN1 staining [47]. Subpopulations of cells, distributed throughout the biofilm, also survive exposure to high concentrations of metal chelating agents [86].

Drug tolerant subpopulations of cells, termed persisters, have been characterized within *Candida* biofilms. These cells survive in the presence of high concentrations of lethal antifungals, but are not resistant since they do not grow in the presence of antimicrobials, harbor stable genetic mutations, or have an increased MIC [49]. Persisters were characterizedby exposing biofilms to high concentrations of amphotericin B or chlorhexidine, and measuring survival. Biphasic killing revealed a subpopulation of cells that were able to tolerate and survive increased drug concentrations. Reinoculation of the cells, which survived killing of the biofilm by amphotericin B produced a new biofilm with a similar subpopulation of persisters, indicating the survivors were not mutants. Persisters were only detected in biofilms, and analysis of biofilm defective mutants suggested attachment was a key mediator of persister formation and not the biofilm per se [49,88]. Persisters were independent of drug

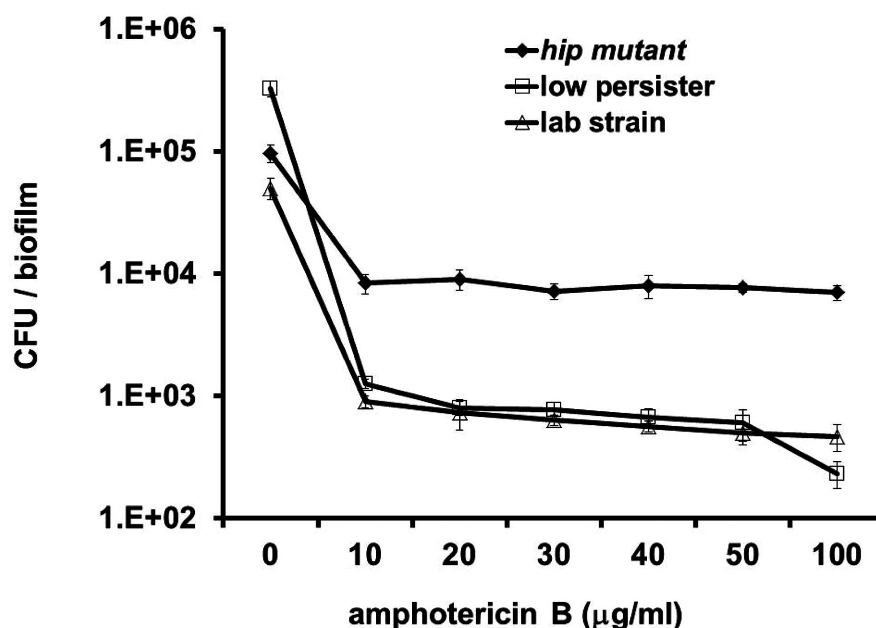

Fig. (1). *C. albicans* strains with increased levels of persisters (hip mutants) were isolated exclusively from cancer patients with long-term oral *Candida* carriage. Biofilms were cultured in microtiter plates and after antifungal challenge, the biofilms were washed, disrupted, diluted, and plated for determination of colony counts (*Copyright © 2010, American Society for Microbiology. Antimicrobial Agents and Chemotherapy, January 2010, p. 39-44, Vol. 54, No. 1*).

efflux and did not have increased MICs to antimicrobials [48,49]. Persisters were also found in biofilms of *Candida krusei* and *Candida parapsilosis* [88]. Biphasic killing and a subpopulation of cells that had increased tolerance to amphotericin B compared to the majority of the cells were also detected in *Candida glabrata*, *Candida tropicalis* and the *C. albicans* laboratory strain SC5314 [88]. The failure to detect survivors in biofilms exposed to 100 µg/mL of amphotericin B does not preclude the presence of persister cells in these strains. *C. albicans* strains isolated from cancer patients undergoing chemotherapy were found to have increased numbers of persisters (Fig. **1**) [48]. The patients in this study received daily chlorhexidine prophylaxis for oral candidiasis, which may have selected for the increased drug tolerance that these strains exhibited. Interestingly, a correlation was found between the presence of high persister strains and the duration of *Candida* carriage within individual patients. These results suggest that persisters are clinically relevant and the presence of persisters within biofilms may be related to the relapse or duration of infections.

The effective treatment of biofilm infected prosthetic devices will likely require a drug that is able to overcome the variety of resistance mechanisms associated with planktonic, surface attached and biofilm growth (Fig. **2**), and is lethal to biofilms and persisters. However, the study of persisters in fungi is partially limited by the fact that most antifungals are static inhibitors of growth. An interesting strategy to overcome the lack of cidal drugs is to identify agents that make growth inhibitory drugs lethal. The calcineurin inhibitors cyclosporine A and FK506 were found to synergize with fluconazole and kill *Candida* cells [89,90]. Calcineurin was subsequently found to be an important mediator of azole drug tolerance and is required for survival during a variety of membrane stresses [91,92]. Hsp90 inhibitors also phenocopy the calcineurin dependent synergy with fluconazole, since calcineurin is a Hsp90 client protein [93,94]. The chaperone Hsp90 is required for survival to a variety of cell wall and cell membrane stressors and therefore Hsp90 depletion renders some drug resistant strains more susceptible to antimicrobials [93,95]. The combination of fluconazole and brefeldin A is also lethal to exponentially growing *Candida* cells and ADP-ribosylation factor cycling represents another mechanism of drug tolerance required for survival to azole antifungals and cell wall stressors [96]. Combinations of fluconazole and cyclosporine A or FK506 were found to be effective in an *in vivo* biofilm catheter model, although the extent of actual killing was not determined [97] and it is unknown whether persisters can survive these combination treatments. Two reports of catheter sterilization have been found. Murkegee *et al.* reported sterilization of catheters after 7 days of antifungal lock with liposomal amphotericin B [98] and Seidler reported a similar effect in a continuous flow model [99]. The failure to detect persisters in these models may be related to the long duration of antifungal exposure or the failure of persisters to remain attached to the surface under the conditions of flow.

CONCLUDING REMARKS

Currently available antifungals are effective against *Candida* in patients for a vast majority of cases. This may be due to the fact that *Candida* largely exits as a commensal, and a modest impact on growth of the pathogen may have larger consequences, tipping the balance of host-pathogen interactions towards the host and against infection. However, the widespread use of antimicrobials and medical devices has led to increased incidences of refractory and recurrent infections.

Failure of currently available antifungal drugs, especially in regard to biofilm infection, suggests a need for the development of new therapeutic strategies. Understanding the mechanisms of biofilm drug tolerance and phenotypic heterogeneity may lead to the development of the first antifungal drugs capable of eradicating infection, salvaging medical devices, and preventing relapse.

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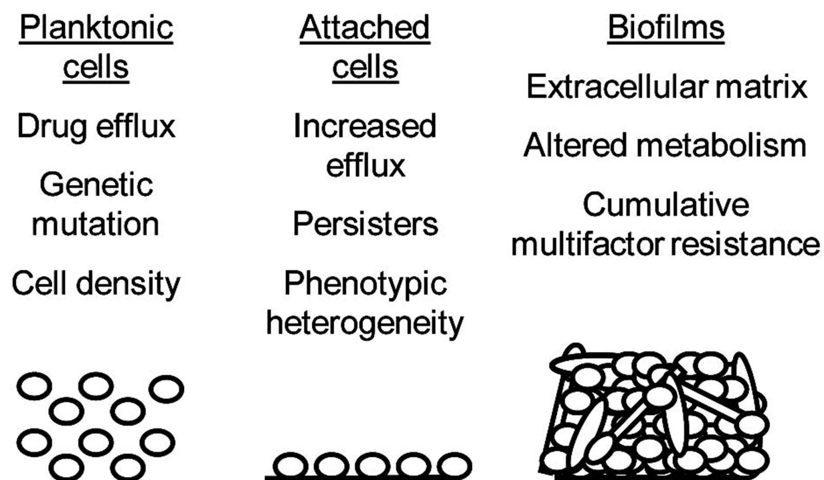

Fig. (2). The mechanisms by which *C. albicans* exhibit resistance to antimicrobials partially depends on whether the cells are planktonic, attached to a surface, or part of a biofilm.

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