Chapter 6
Candida Biofilm Tolerance: Comparison of Planktonic and Biofilm Resistance Mechanisms

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Abstract *Candida* species are opportunistic fungal pathogens residing as commensal organisms in approximately 70% of the human population. During times of decreased immune function, *Candida* spp. are able to transition from harmless members of the human microbiota into pathogens capable of causing life-threatening infections boasting mortality rates as high as 50%. Commonly adhering to implanted medical devices, *Candida* spp. grow as highly structured biofilms with inherent resistance to antifungal drug therapies and the host immune system. A multitude of investigations have found this resistance to be multifactorial involving mechanisms associated with planktonic antifungal resistance (efflux pump activity) along with biofilm-specific mechanisms. One biofilm-specific mechanism involves the complex extracellular matrix. Components of the matrix, specifically β-glucan, mannan, and extracellular DNA, have been found to promote resistance against multiple antifungal drug classes. Here we will review molecular mechanisms contributing to *Candida* biofilm drug resistance.

6.1 Introduction

Candida species is an opportunistic fungal pathogen which exists as a commensal organism in the elementary, gastrointestinal, and genitourinary tract of approximately 70% of the human population (Kabir et al. 2012; Meiller et al. 2009; Rosenbach et al. 2010; Ruhnke and Maschmeyer 2002; Schulze and Sonnenborn 2009; Sobel 1997). In a healthy human, the fungus typically exists in harmony with the normal microbiotic flora of the host. However, in an immunocompromised or immunologically weak host, such as patients receiving chemotherapy, transplant

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recipients, and patients in the intensive care unit, *Candida* is among the most common pathogens and there is risk for spread beyond the mucosa which is associated with mortality in up to half of patients (Pfaller et al. 2005, 2010; Pfaller and Diekema 2007). These lethal cases of candidiasis are often a result of biofilm formation, often on implanted medical devices. In fact, some case series suggest that up to 70% of *Candida* bloodstream infection is linked to biofilm infection of vascular catheters. Considered to be the predominant microbial growth form found in nature, biofilms are an organized community of microbial cells adhered to a surface and enveloped in an extracellular matrix (ECM) with properties that are distinct from their planktonic counterparts (Kolter and Greenberg 2006; Nobile and Johnson 2015). Microbial biofilms exist within environments that are both biotic (aquatic, plant tissues, or mammalian tissues) and abiotic (indwelling medical devices). Microbial species that form biofilms on solid surfaces, such as *Candida* sp., *Staphylococcus* sp., *Streptococcus* sp., and *Escherichia coli,* each form a bio-film with structure, development, and unique properties that are distinct (Nobile and Johnson 2015). Whilst each of these organisms have the capability to form a biofilm on its own, it is becoming clear they often occur in multispecies biofilms due to different species of bacteria and bacterial and fungi that thrive as a result of shared virulence attributes (Roder et al. 2016).

Antimicrobial tolerance is an obstacle to the treatment of numerous biofilm infections (Mah 2012; Romling and Balsalobre 2012). *Candida* biofilms display innate resistance to all available drug classes and withstand antifungal concentrations up to 1000-fold higher than those which are effective toward non-biofilm planktonic cells (Chandra et al. 2001; Donlan and Costerton 2002; Douglas 2003; Ramage et al. 2005). Due to this inherent increased resistance to antifungal drugs, the recommended course of treatment for individuals afflicted with a *Candida* biofilm infection is extirpation of the afflicted device. *Candida* biofilm infections that are not successfully treated have a poor prognosis for the afflicted individual with an associated mortality rate as high as 50% (Al-Fattani and Douglas 2004; Kojic and Darouiche 2004; Mayer et al. 2013; Pfaller and Diekema 2007; Pfaller et al. 2005).

Antifungal resistance is an intrinsic biofilm characteristic and one of the many phenotypic changes that occurs upon transition to this mode of growth (Finkel and Mitchell 2011; Tobudic et al. 2012). During the later phases of development the resistant phenotype is most pronounced, however, drug resistance is able to be detected within minutes to hours of adhesion to a surface (Finkel and Mitchell 2011). Genetic mutations do not account for this observed resistance since biofilm cells re-cultured in planktonic conditions revert back to a susceptibility phenotype to antifungals. Furthermore, it is clear that multiple mechanisms contribute throughout the various stages of biofilm growth to the drug resistance phenotype (Marie and White 2009; Ramage et al. 2012; White et al. 1998a).

6.2 Overview of Antifungal Drug Classes and Planktonic *Candida* Resistance Mechanisms

6.2.1 Triazoles

The triazoles represent the most commonly used of all antifungal classes. Azole antifungal drugs inhibit *Candida* growth by targeting the enzyme lanosterol 14 α-demethylase (encoded by *ERG11*) which is necessary to convert lanosterol to ergosterol. This depletion of ergosterol in the fungal membrane causes the accumulation of toxic sterol intermediates which then lead to growth arrest (Kelly et al. 1995; Kelly et al. 1997; Lupetti et al. 2002; Shimokawa and Nakayama 1992). As with almost all types of antimicrobials, prolonged use has been linked to drug resistance, however, acquired *Candida* drug resistance is relatively uncommon (Cleveland et al. 2012; Ostrosky-Zeichner et al. 2010; White et al. 1998a). Long-term treatment of oral or esophageal candidiasis as reported in HIV/AIDS, especially in the pre-HAART era (Law et al. 1994; White et al. 1998a) was associated with high rates of resistance. The reported mechanisms of *C. albicans* azole resistance have included *ERG11* point mutations (S405F, Y132H, R467 K, and G464S), gene amplifications, and mitotic recombination events within *ERG11* or the drug efflux pumps (*Cdr1p, Cdr2p, and Mdr1p)* which result in their increased expression (Albertson et al. 1996; Coste et al. 2007; Lamb et al. 1997; Marichal et al. 1999; Sanglard et al. 1998; Sanglard et al. 1995; vanden Bossche et al. 1992; White 1997a, b). Acquired resistance in *C. glabrata* is even more common. Conversely, resistance in *C. parapsilosis* and *C. tropicalis* is uncommon (Bizerra et al. 2008; Cannon et al. 2009; Silva et al. 2011; vanden Bossche et al. 1992; Vandeputte et al. 2005). However, intrinsic resistance during biofilm growth is universal among all *Candida* species. Cells within a biofilm environment tolerate 1,000-fold higher azole concentrations than their planktonic counterparts, acquiring resistance as early as 4–6 h after initial adherence to a surface, resulting in ineffective triazole treatment (Lamfon et al. 2004; Ramage et al. 2001, 2012).

6.2.2 Polyenes

The polyene antifungals were the first available for systemic therapy. These Polyenes are amphiphilic molecules allowing for binding with sterols, primarily ergosterol, in the fungal cell membrane. This binding alters the transition temperature of the cell membrane, decreasing membrane fluidity and permeability. Release of monovalent ions (K^+, Na^+, H^+, and Cl^-) and small organic molecules ultimately leads to death of cell (Baginski and Czub 2009; Gray et al. 2012). Amphotericin B is a potent antifungal but is reserved for patients with severe systemic fungal infections due to the severe and potentially lethal side effects, the most important of which is renal toxicity. Resistance to amphotericin B is rare, but has been described

in case reports from cancer patients undergoing chemotherapy and individuals undergoing prolonged prophylactic therapy (White et al. 1998a). The specific mechanisms of acquired polyene resistance are mechanistically poorly defined, but thought to involve alterations to cell membrane composition. Resistance has been linked to sterol changes in *C. glabrata* (Vandeputte et al. 2007) as well as in genetically altered strains that are defective in sterol C5,6—desaturase which produce little ergosterol (Kelly et al. 1996; Kelly et al. 1997). As with the triazoles, biofilms exhibit resistance to amphotericin B as well, but only 10–100 times as much as their planktonic counterparts in comparison to the 1,000 fold greater resistance associated with triazoles (Tobudic et al. 2010). Unfortunately, the concentrations needed for effective therapy are not achievable during systemic administration.

6.2.3 Echinocandins

The echinocandins represent the most recently available antifungal drug class. They act via inhibition of β-1,3 glucan synthase which is a key component of *Candida* cell walls (Denning 2003; Perlin 2015b; Shapiro et al. 2011). Inhibition of this enzyme at the cell wall results in osmotic instability within the cell ultimately leading to lysis of the cell (Chaffin et al. 1998). Clinical trial results suggest superiority over other antifungals for invasive candidiasis, likely due to its cidal activity and relative safety of this drug class. While, resistance remains relatively uncommon among all *Candida* species, treatment failures among *C. glabrata* have been emerging more rapidly. Point mutations along "hot spot" regions of the Fks1 subunits, located specifically among the range of amino acids from Phe641 to Pro649 and Arg1361, are the most commonly seen mechanisms of acquired resistance among this class of drugs and have been observed in *C. albicans* and homologous regions in *C. glabrata FKS2* gene (Balashov et al. 2006; Desnos-Ollivier et al. 2008; Garcia-Effron et al. 2009; Hernandez et al. 2004; Johnson et al. 2011; Katiyar and Edlind 2009; Laverdiere et al. 2006; Park et al. 2005; Perlin 2007, 2015a, b; Shapiro et al. 2011). As with the other antifungal drug classes, biofilms are intrinsically more resistant to echinocandins than their planktonic counterparts by approximately 2–20 fold (Nett et al. 2010a; Tobudic et al. 2010).

6.2.4 5-FC/Flucytosine

Flucytosine is a pyrimidine analogue that is metabolized in the pyrimidine salvage pathway by a cytosine deaminase into a toxic version of UTP. Upon incorporation, RNA synthesis is halted (Hope et al. 2004; White et al. 1998b). Flucytosine also decreases the availability of nucleotides for DNA synthesis via conversion into a

metabolite that inhibits thymidylate synthetase (Hope et al. 2004). Emergence of resistance with flucytosine monotherapy is relatively rapid. This resistance is due to mutations in the cytosine permease gene *FCY2,* which is responsible for escorting flucytosine into the cell, or in the cytosine deaminase gene *FCY1* (White et al. 1998b). Due to this rapid rate of acquired resistance, flucytosine is almost generally administered to patients in conjunction with amphotericin B and/or azole antifungals (Pappas et al. 2009).

6.3 *Candida* Biofilm Resistance Mechanisms

Candida biofilms have been the subject of numerous investigations. Initial studies into the mechanisms of drug resistance primarily explored mechanisms previously linked to drug tolerance in planktonic cells. Mutations in genes that encode drug target enzymes, such as *ERG11* and *FKS1*and alterations in the composition of the plasma membrane have all been linked to planktonic cell resistance but have not been demonstrated to contribute to biofilm resistance (Balashov et al. 2006; Bizerra et al. 2011; Morschhauser 2002; Stevens et al. 2006).

6.3.1 *Role of Efflux Pumps*

As described above, the overexpression of efflux pumps, coupled with the reduction of antifungal accumulation within the cell, is a key mechanism of resistance for planktonic *Candida* (Morschhauser 2002). Ramage et al. examined if upregulation of efflux pumps may also contribute drug tolerance during biofilm growth. They found increased transcription of both *MDR1* and *CDR1* in 24 h *C. albicans* biofilms when compared to their planktonic counterparts of the same growth stage (Ramage et al. 2002a). Genetic manipulation, via deletion, of *MDR1, CDR1,* and/or *CDR2* was conducted in order to investigate the role of efflux pumps on triazole resistance during biofilm growth. Hypersensitivity to fluconazole was displayed by these mutants during both planktonic and biofilm growth, but not in biofilms grown for 24–48 h of the same mutant strain. This suggested that during the mature biofilm stage efflux pumps do not significantly contribute to drug resistance (Ramage et al. 2002a).

Mukherjee et al. explored the impact of efflux pumps at three phases of biofilm development including early (0–11 h), intermediate (12–30 h), and mature (31–72 h) time points in comparison to their planktonic counterparts (Mukherjee et al. 2003). Single, double, and triple mutants of these three main efflux pump genes showed no increase in susceptibility to fluconazole during the mature biofilm growth phase; however, in the early phase (6 h) the double and triple efflux pump mutants displayed a modest increase in azole susceptibility as compared to the parent strains (Mukherjee et al. 2003). Loss of a single efflux pump had little to no

effect in regards to biofilm resistance, even at the earliest time points as seen in the double and triple mutants. This suggested that not only do efflux pumps function in a cooperative manner, but that they also contribute to resistance during the early biofilm developmental stages opposed to the later mature stages. To further investigate this observation, transcriptional analysis of efflux pump genes were conducted on 12 and 48 h biofilms which found with elevated expression levels of said genes during the earlier less mature phase in comparison to the older mature phase (Mukherjee et al. 2003). Additional studies directed at *C. glabrata* and *C. tropicalis* also suggested efflux pumps more than likely contribute to biofilm drug resistance during the early phases of growth (Bizerra et al. 2008; Ramage et al. 2012).

6.3.2 Influence of Sterol Synthesis

Mukherjee et al. explored the role of plasma membrane changes during biofilm formation, as changes in sterol synthesis have been linked to amphotericin B and ergosterol resistance during planktonic growth, as described above (Ghannoum and Rice 1999; Kontoyiannis 2000; Mukherjee et al. 2003). Initial studies examined the levels of sterols during different stages of biofilm development and found that early phase biofilms contained relatively similar levels of ergosterol as that of time matched planktonic cells. However, as biofilm development continued, the ergosterol levels reduced to 50% of the levels measured for planktonic conditions (Mukherjee et al. 2003). Furthermore, the sterol profile of intermediate and mature biofilms was different than planktonic cultures of the same age. Specifically, concentrations of ergosterol decreased as the biofilms aged and were replaced by intermediate sterols such as zymosterol, 4,14-dimethylzymosterol, and obtusifoliol (Mukherjee et al. 2003). This finding suggested that during the early stages of biofilm development ergosterol is an effective target for drug therapy, but as biofilms continue to grow and mature their dependency upon ergosterol decreases, potentially limiting the efficacy azole and polyene antifungals which target ergosterol.

Global transcriptional analysis also showed increased levels of *ERG11* transcription during the early phase growth stage of *C. albicans* biofilms in comparison to planktonic cells of the same age (Finkel and Mitchell 2011; Nett et al. 2009). A second gene that plays a role in ergosterol biosynthesis, *ERG25,* was found to be upregulated in intermediate and mature biofilms when compared to planktonic cultures of the same age (Nett et al. 2009). *ERG25* encodes a putative C-4 sterol methyl oxidase which is believed to play a role in the biosynthesis of ergosterol intermediates via C4-demethylation (Nett et al. 2009). The conversion of lanosterol to nonergosterol intermediates, such as eburicol and 14-methyl fecosterol, is a role that this enzyme is theorized to perform in biofilms. Additional studies directly testing the role of membrane changes in the biofilm drug-resistant phenotype have not been reported.

6.3.3   Impact of Cell Density and Quorum Sensing

Another trait contributing to the enhanced drug resistance observed during biofilm growth is the relatively large fungal burden (Perumal et al. 2007; Seneviratne et al. 2008). This relationship has also been described for planktonic cells, with higher inoculums producing higher MICs (Nguyen and Yu 1999; Riesselman et al. 2000). Based upon these studies, Perumal et al. examined the role of high cell density on antifungal resistance by comparing the susceptibility levels of planktonic yeast cultures with those of intact and disrupted biofilms (Perumal et al. 2007). Similar to findings in planktonic cells, high cell density cultures displayed higher levels of resistance to azoles when compared to cultures of lower density in the biofilm state.

Quorum sensing is the signaling process linked to control of cell density in the biofilm state. Two key quorum sensing molecules, tyrosol and farnesol, have opposing roles during biofilm development. Tyrosol promotes the hyphal state of biofilms whereas farnesol promotes the yeast state (Hornby 2001; Lindsay et al. 2012; Ramage et al. 2002b; Wongsuk et al. 2016). In addition to the roles of fungal morphogenesis and fungal development, quorum sensing molecules have also been implicated as having potential antifungal activity. Studies in which biofilm cells are co-treated with antifungal drugs (azoles and polyenes) and quorum sensing molecules have demonstrated a synergistic effect (Sharma and Prasad 2011; Wongsuk et al. 2016). Sharma et al. found that farnesol is able to reduce drug extrusion, of azoles, through the ABC transporters CaCdr1p and CaCdr2p, which may in part explain the synergistic effect observed with a triazole (Sharma and Prasad 2011).

6.3.4   Contribution of Biofilm Extracellular Matrix

The production of extracellular matrix is a distinctive feature of biofilms (Mitchell et al. 2015; Zarnowski et al. 2014). This matrix encompasses the cells within the biofilm and promotes cohesion among the cells within as well as adhesion to surfaces (Flemming and Wingender 2010; O'Toole 2003). Furthermore, the matrices of most microbes have been found capable of absorption numerous environmental components. For example, the surrounding matrix has been shown to retain water and nutrients (Flemming and Wingender 2010). However, the most studied aspect of the extracellular matrix is its ability to create a protective physical barrier between biofilm cells and its surrounding environment. This proves to be vital to the organism's survival when growing on the surface of an indwelling device by providing protection from pharmacological agents and the hosts innate immune system (Costerton et al. 1999; Donlan 2001).

The Douglas group performed the first studies on fungal biofilms using *C. albicans* to investigate the potential role of the extracellular matrix on drug resistance and the importance of environmental conditions on overall matrix production

(Douglas 2003). Since this initial study a number of labs have investigated the matrix composition and role in drug resistance in fungal biofilms. A recent detailed analysis of the *C. albicans* matrix identified each of the four macromolecules classes. Relative composition of the matrix based upon dry weight included 55% protein, 25% carbohydrate, 15% lipid, and 5% nucleic acid (Zarnowski et al. 2014). Proteomic analysis revealed 458 distinct entries which included protein classes involved in carbohydrate and amino acid metabolism (Al-Fattani and Douglas 2006; Faria-Oliveira et al. 2014; Thomas et al. 2006). Analysis of the carbohydrate fraction revealed the presence of three polysaccharides, β-1,3 glucan, β-1,6 glucan, and α-1,6 mannan with α-1,2 linked branches. Identified lipids included neutral and polar glycerolipids in addition to a small portion of sphingolipids (Zarnowski et al. 2014). The nucleic acids found consisted mainly of noncoding sequences of DNA (Zarnowski et al. 2014).

Nett et al. examined the relationship between biofilm resistance and matrix by taking purified matrix material from biofilms and adding it to planktonic cells prior to antifungal susceptibility testing (Nett et al. 2007). The addition of matrix rendered the planktonic cells resistant to antifungal drug to a degree that was similar to that seen in mature biofilms. This suggested that matrix material is interacting or sequestering antifungals preventing them from reaching their intended targets. Using radiolabeled fluconazole, they found that cultures containing matrix were able to sequester the azole drug from their intended targets, consistent with this theory. Genetic studies seeking the component responsible for this process implicated the carbohydrate β-1,3 glucan (Nett et al. 2007, 2010b). This drug sequestration phenomenon has been found to relatively nonspecific with regard to the antifungal drug as resistance linked to matrix protection has been shown important for triazoles, polyenes, flucytosine, and echinocandins (Nett et al. 2010a; Vediyappan et al. 2010). Studies conducted on biofilms formed by other *Candida* spp., namely *C. glabrata, C. parapsilosis,* and *C. tropicalis* also displayed this matrix antifungal mechanism as well as affinity toward β-1,3 glucans role in biofilm resistance (Fernandes et al. 2015; Kuhn et al. 2002; Mitchell et al. 2013; Nett et al. 2010a; Yi et al. 2011).

The first investigation into the genetic control of production of matrix β-1,3 glucan have shown that the β-1,3 glucan synthase Fks1p is required for production of this polysaccharide (Nett et al. 2010a). Subsequent delivery to the matrix has been found to be regulated in a complementary fashion by three glucan modifier proteins Bgl2p, Phr1p, and Xog1p (Nett et al. 2010a; Nett et al. 2010b; Taff et al. 2012). Additionally, Nobile et al. identified a transcription factor, Zap1p, that is critical for control of matrix production (Nobile et al. 2009). Zap1p was found to negatively regulate production of β-1,3 glucan by hydrolysis of matrix carbohydrates through the control of two glucoamylases *GCA1* and *GCA2*. It is also speculated to influence matrix production through quorum sensing pathways based upon its control over alcohol dehydrogenases ADH5, CSH1, and LFD6 enzymes (Nobile et al. 2009).

Recent work has also shown that the drug resistance phenotype is not solely due to β-1,3 glucan. In fact, each of the three polysaccharides identified in the

carbohydrate fraction of the extracellular matrix were found to cooperate as a mannan-glucan complex to facilitate drug resistance in *C. albicans* biofilm. Mitchell et al. further investigated the genetic implications of the loss of genes which regulate production and modification of mannan and glucan within the extracellular matrix. They found that a subset of mutants had reduced levels of all three polysaccharides, lower levels of total matrix, and increased susceptibility toward antifungals. The β-1,3 glucan synthase gene (*FKS1),* two genes regulating matrix β-1,6 glucan (*BIG1* and *KRE5*), and seven genes regulating matrix mannan (*ALG11, MNN4-4, MNN9, PMR1, VAN1,* and *VRG4*) all prominently displayed the aforementioned phenotype. Surprisingly though when biofilms containing mutants from the various pathways were mixed and grown with one another, matrix structure and functionality was restored (Mitchell et al. 2015). This observation in addition to studies pharmacologically manipulating the matrix components demonstrated that the matrix constituents were assembled after export from the cell.

Extracellular DNA (eDNA) has also been found to impact the drug resistance phenotype seen in biofilms. This finding is based upon investigations that showing an increase in susceptibility of *C. albicans* biofilms to two classes of antifungals (echinocandins and polyenes) when co-treated with DNase (Martins et al. 2012; Martins et al. 2010). It is still unclear how the eDNA is mechanistically contributing to drug resistance (Zarnowski et al. 2014).

6.3.5 *Presence of Persister Cells*

Persister cells are a subpopulation of dormant cells found within biofilms, which exhibit a higher tolerance to multiple drug classes (LaFleur et al. 2006; Lewis 2008). They are defined as the population of microbes remaining after antimicrobial exposure and were first described for amphotericin B. The cells are capable of reconstituting new biofilms containing the same percentage of resistant persister cells (0.01–0.02%). These characteristics suggest they serve as the primary component of recurring fungal infections (Sun et al. 2016). Although the underlying mechanism for the production of persister cells is still unclear, it linked surface adhesion. Sun et al. showed they rapidly emerged upon surface adhesion, reaching a state of saturation by 2 h (Sun et al. 2016). The genetic basis underlying the persister cell lifestyle remains unclear.

6.4 Conclusion

The ability of *Candida* spp. to transition into a biofilm lifestyle allows this organism to thrive in even the healthy human host. This survival is attributed to the intrinsically high levels tolerance of conventional antifungal therapies and the host's

innate immune response. The process appears multifactorial with resistance mechanisms varying by the phase of biofilm development.

Efflux pumps play an important role during the early phase of development. This mechanism of resistance is most prevalent during the stage at which the planktonic cells begin to adhere to a surface and transition into a biofilm state. As the biofilm matures the role played by these efflux pumps diminishes and instead biofilm-specific mechanisms are predominant. The earliest biofilm-specific mechanism is the production of persister cells, which are phenotypic variants of the parent cells that are resistant to antifungals and provide a mechanism for regrowth of the organism after high levels of drug exposure. As biofilms continue to mature an extracellular matrix is produced which contains a multitude of components that work together to provide multilayers of protection to the cells which now reside within this material. Carbohydrates of the matrix, specifically β-1,3 glucan, β-1,6 glucan, and α-1,6 mannan with α-1,2 linked branches, sequester a variety of antifungals providing drug resistance to mature biofilms. A secondary component,
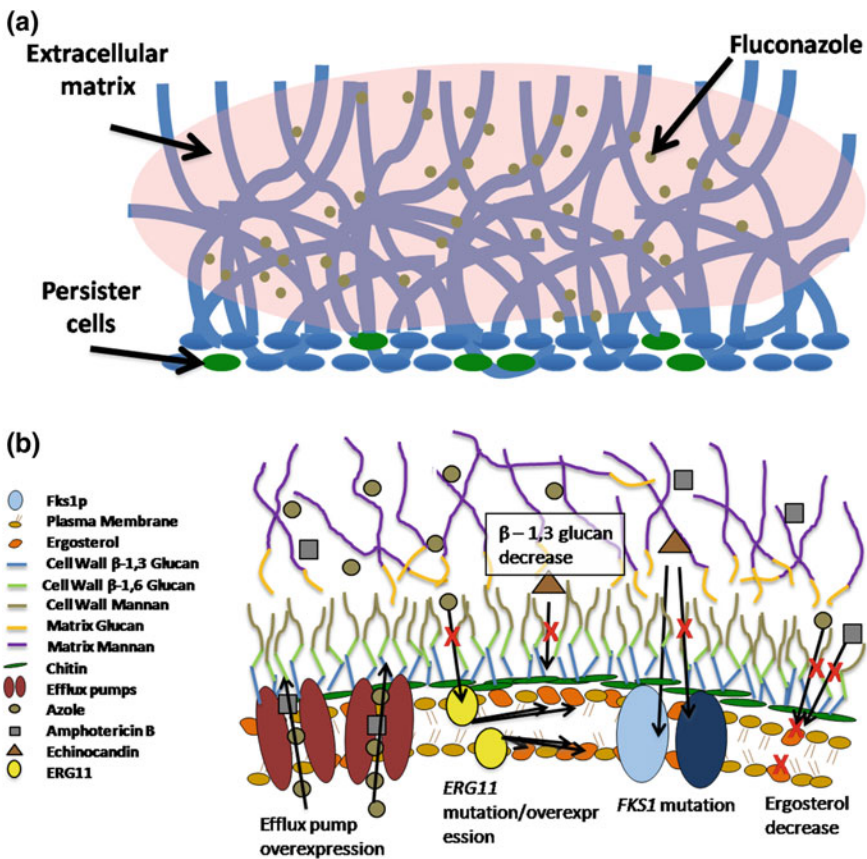

Fig. 6.1 *Candida* biofilm resistance mechanisms. **a** Resistance mechanisms at the biofilm community level. **b** Resistance mechanisms at the cellular level (Taff et al. 2013)

extracellular DNA, also promotes drug resistance to popular antifungals further contributing to this phenotype.

Biofilms contain many overlapping and redundant mechanisms which allow them to survive in hostile environments and evade drug treatments resulting in poor prognosis for patients (Fig. 6.1; Taff et al. 2013). Compositional, structural, and biochemical analysis of biofilms and their components have allowed us to better understand this complex organism and potentially develop innovative therapies to better combat infections. However, there are still many unknowns, such as the role of host factors and their interaction with matrix components during infection or do the components of duel species biofilms interact with one another to further enhance drug resistance? Additional investigations addressing questions such as these are still necessary in order to fully comprehend the nature and full potential of fungal biofilms.

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