Relating assembly and function of the *Candida albicans* biofilm extracellular matrix

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As a biofilm, microbes produce an extracellular matrix that confers protection from the surrounding environment. For pathogenic microorganisms, this protection is manifested as high levels of drug resistance, making biofilm-related infections incredibly difficult to eradicate. Elucidation of matrix biogenesis mechanisms thus addresses an interesting biological question as well as an urgent medical need.

The most common hospital-acquired fungal pathogen, *Candida albicans*, frequently forms biofilms on implanted medical devices, often leading to lethal disseminated disease. The intrinsic resistance of biofilms is multi-factorial, but is due largely to the extracellular matrix encasing the biofilm cells. Prior studies indicate that the matrix is complex, with major polysaccharide constituents α-mannan, β-1,6 glucan, and β-1,3 glucan.

This work implemented multiple approaches to unravel the contributions of the three polysaccharides to matrix structure and function. Inhibiting synthesis of any one polysaccharide altered quantities of the others. Each polysaccharide was also required for matrix function, as assessed by assays for antifungal sequestration. The role of these matrix polysaccharides in drug resistance is similar for other clinically-relevant *Candida* species, suggesting the possibility of conserved matrix structure and function.

These results indicate that matrix biogenesis entails coordinated delivery and assembly of the individual polysaccharides. To ask whether this occurs inside or outside the biofilm cells, matrix-defective mutant strains were evaluated for functional matrix production in biofilm co-culture. These mixed biofilms, inoculated with mutants containing a disruption in each

polysaccharide pathway, had restored mature matrix structure, composition, and biofilm drug resistance. These results argue that functional matrix biogenesis is coordinated extracellularly, and thus reflects the cooperative actions of the biofilm community.

A mechanism for the delivery of matrix materials by extracellular vesicles (EVs) is also newly defined here. Variants of EVs have been identified in cell types across biological domains, with diverse functions including unconventional secretion of macromolecules and intercellular signaling. EVs were isolated from *C. albicans* biofilms, and found to contain carbohydrates as well as proteins involved in polysaccharide remodeling. Mutant biofilms deficient in EV production had increased drug susceptibility, supporting the notion that these structures are important for the accumulation and extracellular assembly of functional matrix.

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Chapter 1: Literature Review

Section 1: Clinical Relevance of *Candida* Biofilm Infections

Mucosal *Candida* Infections

Candida albicans is a commensal fungus in roughly 80% of humans, typically found in the gastrointestinal and genitourinary tracts [1, 2]. While normally harmless, this fungus has the ability to infect numerous tissues when conditions in the body are altered. Change in immune status or treatment with broad-spectrum antibiotics can permit *Candida* to flourish.

The most common infections are mucosal: oral candidiasis (also known as thrush), vulvovaginal candidiasis (commonly referred to as yeast infection), and cutaneous candidiasis (or diaper rash) [2, 3]. Vulvovaginal candidiasis is relatively common, and nearly 75% of all adult women will have at least one infection in their lifetime [4]. Thrush often occurs in AIDS patients, with up to 90% experiencing at least one episode [3]. Mucosal *C. albicans* infections can typically be effectively treated with azole antifungals [5]. Acquired resistance to this drug class is rare, and seen primarily in patients on long-term prophylaxis such as those with HIV [6, 7].

Bloodstream and Invasive Candidiasis

Less common than mucosal infections, though much more difficult to treat, are bloodstream and disseminated forms of candidiasis. Risk factors for *Candida* bloodstream infections (BSIs) include immunosuppression, introduction of a foreign medical device, broad-spectrum antibiotic use, mechanical ventilation, renal replacement surgery, and stays in the ICU [8, 9]. Of all *Candida* species, *C. albicans* is the most common cause of infection. Incidence rates vary greatly depending on the population and location of study, but nearly all *Candida* infections are consistently caused by four species in addition to *C. albicans*: *C. parapsilosis*, *C. glabrata*, *C. tropicalis*, and *C. krusei* [10]. *C. albicans* is currently ranked as the 4th most common nosocomial bloodstream infection, with resulting disseminated infections estimated to have up to 40% mortality [1, 11].

Echinocandins are recommended for treatment of candidemia, with possible transition to fluconazole after several days [8, 12]. Fluconazole can be used as an alternative for initial therapy

in patients who are not critically ill and are not likely to have azole resistance. Azole susceptibility testing is recommended for all bloodstream isolates, and antifungal treatment should last 2 weeks after the last negative bloodstream culture.

Device-associated infections

Though *Candida albicans* has clearly coevolved with its human host in a commensal relationship, relatively recent developments in medicine have increased opportunities for its pathogenicity [13-15] (**Figure 1**). Medical devices have become an essential component of modern patient care, with an estimated 45 million devices implanted in the US each year [16]. These are ready substrates for biofilm infection by microbes such as *Candida*, and the NIH estimating that 80% of human infections are the result of pathogenic biofilms [17]. Devices at risk include catheters, cardiac valves, pacemakers, prosthetic joints, and dentures. Device infection can be life threatening: of the 5 million central venous catheters (CVCs) placed in the US each year, 12% become infected, leading to 20,000 deaths [18]. Risk factors for CVC-related infection include duration of site use, total parenteral nutrition, or improper catheter insertion [1, 19]. Strikingly, up to 80% of *Candida* BSIs stem from biofilms formed on CVCs [1, 8].

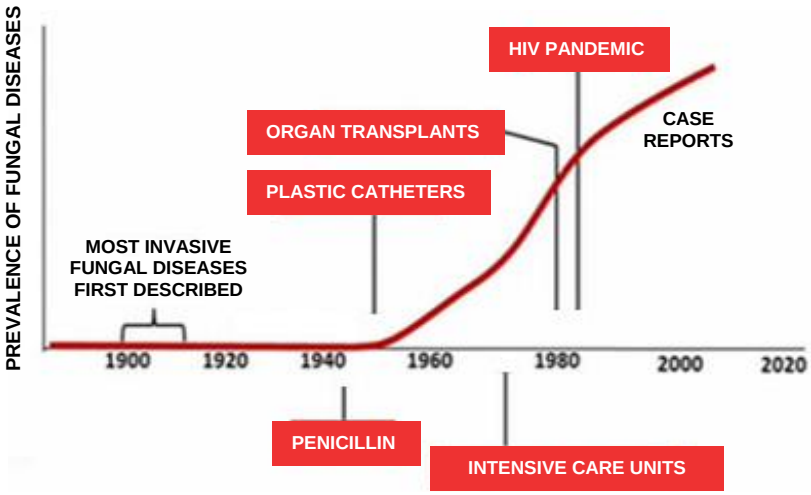

Figure 1. Notable events in the 20th century that have aided the rise in prevalence of fungal pathogens. Adapted from Arturo Casadevall, 2010 (reference 13).

Catheters can become infected by several routes. In neutropenic patients, yeast can traverse the lining of the gut to enter the bloodstream and eventually seed the catheter. Another source can be the hands of hospital staff or patients, allowing *Candida* cells to travel along the catheter and into the bloodstream [1, 20, 21]. Rarely, the catheter infusate itself can be infected [21]. Formation of *C. albicans* biofilms begins with attachment of yeast-form cells to the device surface, which multiply to form a basal layer of yeast cells. This initiates upregulation of biofilm-specific processes, namely the development of hyphae, co-aggregation with other cells, intercellular communication, and extracellular matrix production [22-25]. The resulting community is a heterogeneous mix of hyphae, pseudohyphae, and yeast cells, and can often form as a mixed-species biofilm with other microbes. Hyphal cells near the top of the mature biofilm undergo budding, and the new cells (mostly yeast) disperse away from the biofilm. This dispersion is what constitutes BSI and subsequent disseminated disease [26]. There are currently no effective drug therapies in practice for biofilm elimination. In most cases the medical device harboring a biofilm must be replaced, creating huge cost and risk for patients [5, 12, 27].

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Chapter 1: Literature Review

Section 2: 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 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. Here we will review the molecular mechanisms underlying *Candida* biofilm antifungal resistance and their relative contributions during various growth phases.

KEYWORDS: *Candida*, biofilm, antifungal resistance, extracellular matrix, glucan, persister cells, calcineurin, efflux pumps

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, multi-drug 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 post 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 them from reaching their cellular targets. This mechanism has been linked to production and delivery of β-1,3 glucan to the matrix.
- *C. 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 mitogen-activated protein kinase 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 activity of some antifungals.

Conclusions

- 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.

Introduction

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 non-biofilm, planktonic cells [4-7]. Because common drug therapies do not eradicate *Candida* biofilms, removal of the infected device is almost always necessary for cure of 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 mortality. Many of the pioneering biofilm investigations have focused on *Candida albicans* [14-16]. However, it is becoming increasingly clear that numerous *Candida* spp., including *C. glabrata*, *C. parapsilosis, C. dubliniensis*, and *C. 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 re-cultured 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* 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 and imidazole antifungal drugs inhibit *Candida albicans* by targeting the lanosterol demethylase encoded by *ERG11*. This disrupts synthesis of ergosterol, causing an increase in the 14α-methyl sterol precursors in the cell membrane, and 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 oroesophageal candidiasis pre-HAART [18, 27]. Reported mechanisms of *C. albicans* azole resistance include mutations resulting in increased expression of efflux pumps (*CDR1*, *CDR2,* and *MDR1*) 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 been shown to play a role in azole resistance in *C. parapsilosis*, *C. krusei*, *C. tropicalis,* and *C. glabrata* as well [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 1,000 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, thus forming pores that disrupt the cells' membrane gradients [25]. Although two of the less common *Candida* species, *C. lusitaniae* and *C. guillermondii*, show intrinsic amphotericin

B resistance, acquired resistance to amphotericin B is relatively rare. Resistance has been reported in 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, 44]. A similar mechanism of resistance has been reported for *Candida glabrata* strains as well [45]. Biofilms are around 8 times more resistant to amphotericin B than their planktonic counterparts, depending on the age of the biofilm [46]. 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 *Candida albicans* gene *FKS1* [47-49]; 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 [50]. Resistance to echinocandins has been linked to acquired or intrinsic *FKS1* point mutations in *C. albicans* [48, 49, 51-55]. Similar mutations have been described for echinocandin-resistant *Candida tropicalis, C. glabrata,* and *Candida krusei* strains [53, 56]. 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 [57, 58]. However, the in vivo significance of this resistance mechanism is unclear. Compared to planktonic cells, biofilms are approximately 2-20-fold more resistant [59] .

5-FC/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 UTP that becomes incorporated into RNA and halts its synthesis [18, 60]. Flucytosine is also converted into a metabolite that inhibits the thymidylate synthetase and thus decreases the availability of nucleotides for DNA synthesis [60]**.** A study in 1980 claimed that up to 33% of *Candida* strains in the US were inherently flucytosine resistant, which is one reason its use has been limited; more recent data puts this frequency of resistance between 3 and 8% [60, 61]. Non-*albicans* spp. of *Candida*, and the *C. albicans* B serotype seem to have the higher rates of intrinsic resistance [18, 62]. 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 [63]**.**

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* [52, 64]. In addition, alterations in the composition of both the cell membrane and the cell wall have been linked to antifungal resistance in non-biofilm cells [57, 58, 64]. 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 [64]. In *Candida albicans***,** efflux pumps have primarily been described as playing a role in azole resistance, but not in resistance to amphotericin B or the echinocandins [49, 65-67]. The ABC transporters (*CDR1* and *CDR2*) and major facilitator transporter (*MDR1)* are typically expressed at low levels in the absence of antifungal exposure [68]. The finding that azole-resistant clinical isolates often show constitutive over-expression of these pumps prompted investigators to postulate that the biofilm drug resistance phenotype may be related to increased efflux pump activity [32-34, 68-70].

It was demonstrated by Ramage et al. that transcription of both *MDR1* and *CDR1* was more abundant in 24 hr *C. albicans* biofilms than planktonic cultures of the same age [71]. 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 hrs, suggesting that the efflux pumps do not contribute significantly to drug resistance during the mature biofilm stage [71].

Mukherjee et al. examined the role of efflux pumps in antifungal resistance throughout the biofilm process [72]. They included early (0 – 11 hr), intermediate (12 – 30 hr), and mature (31 – 72 hr) *C. albicans* biofilms with planktonic growth comparisons. Similar to the prior investigation, single, double, and triple mutants of the 3 main efflux genes were no more susceptible to fluconazole treatment during mature biofilm growth than the parent strains; however, in the early

phase (6 hr), double and triple efflux pump mutants had significantly increased azole susceptibility when compared to the parent strains [72]. 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 hr of biofilm formation when compared to mature, 48 hr biofilm formation [72].

Increased expression of efflux pumps during biofilm growth has been confirmed in a rat venous catheter model when compared to planktonic cultures of the same age, including *CDR2* at 12 hrs and *MDR1* at 12 and 24 hrs [73]. 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 [72]. Mukherjee et al. examined the sterol composition in biofilms during the various phases of biofilm formation [72]. 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 [72]. 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 against them.

Several studies examining the various phases of biofilm formation have shown alterations in the transcriptional profile of sterol pathway genes [8, 73]. Microarray analysis demonstrated increased transcription of both *ERG25* and *ERG11* during in vitro *C. albicans* biofilm growth when compared to planktonic cells of the same age. *ERG11* encodes the azole drug target and *ERG25* encodes a putative C-4 methyl sterol oxidase, thought to play a role in C4-demethylation of ergosterol biosynthesis intermediates. 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 [73]. Interestingly, transcriptional analysis of a rat venous catheter biofilm also found increased transcription of *ERG25*, but not *ERG11* [73]. Differential expression of ergosterol synthesis genes has also been described for *C. parapsilosis* and *C. dubliniensis* [20]. 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 biofilm cells deep in a biofilm grow more slowly due 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 [74, 75]. By controlling nutrients in a perfused biofilm fermentor, Baillie and Douglas were able to compare the antifungal susceptibility of biofilms growing at various rates [76, 77]. 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 and quorum sensing

Another biofilm-specific trait suspected to influence drug susceptibility is the relatively high concentration of fungal cells in a biofilm compared to many planktonic conditions [78, 79]. To examine the role of high cell density on antifungal resistance, Perumal et al. compared the susceptibility of planktonic yeast cultures with intact and disrupted biofilms [78]. Cells from each of these conditions showed greater resistance to azole drugs at a high cell density than at a low cell density. This finding suggests that the high cell density of biofilms influences antifungal resistance, but that is not an attribute that applies to only biofilm cells.

Quorum sensing and cell density are closely linked in *Candida* biofilms. Two key quorum sensing molecules in *Candida albicans*, tyrosol and farnesol, exert opposing activities [80-82]. Farnesol has been shown to inhibit both biofilm formation and the development of hyphae [82, 83]. This effect has been observed for *C. albicans* biofilms as well as *C. dubliniensis* [84, 85]. Microarray analysis of farnesol-treated biofilms first suggested a link between quorum sensing and drug resistance [86]. Biofilms exposed to farnesol were found to have decreased transcription of *PDR16*, an ABC 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 lacking a known regulatory component and defective in farnesol response does not demonstrate increased fluconazole susceptibility in high density conditions [78]**.**

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 [83, 87].

Investigations have questioned if the biofilm matrix material may also impair drug delivery, either via steric hindrance or by actively binding or sequestrating antifungals. The Douglas group performed the first studies in this realm. By altering growth conditions, they were able to produce biofilms with different quantities of extracellular matrix. Compared to 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 [88]. 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, were they able to link *Candida* biofilm resistance to the production of an extracellular matrix [89]. A correlation between the degree of extracellular matrix production and antifungal resistance was also found for *C. tropicalis* biofilms [89].

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 [90]. The observation that addition of biofilm matrix to non-biofilm 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 drug. This resistance appears to correlate with production of a matrix carbohydrate, β-1,3 glucan [90-92]. Vediyappan et al. found that biofilm matrix also interacts with amphotericin B, and implicated this interaction in biofilm drug resistance [93]. Additional studies support a similar biofilm resistance mechanism for a variety of antifungals, including flucytosine, and the echinocandin drug class [94]. Biofilms formed by other *Candida* spp., including *C. glabrata, C. parapsilosis,* and *C. tropicalis*, also display a matrix-antifungal sequestration mechanism of drug resistance [95]. Compared to other available antifungals, the echinocandin drug class appears to have more anti-biofilm activity. One explanation for this is that these drugs inhibit β - 1,3 glucan synthesis, possibly impairing production of the critical matrix component [96].

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 the biofilm drug resistant phenotype both in vitro and in an animal model [91]. This pathway appears to be under control of regulator Smi1 through the transcription factor Rlm1 [97]. 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 [98]. 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) [92]. 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 (eDNA) [99, 100]. This is based on the finding that treatment of *C. albicans* biofilms with DNase augments the activities of echinocandins and polyenes [100]. 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 [101, 102] but a similar role in *Candida* has

not been described [3, 103, 104]. It is unclear how matrix eDNA 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 [105, 106]. These dormant variants can serve as the inoculum for a new biofilm containing the same percentage of persister cells [105]. 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 [107]. 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 vsubpopulation, while it is suggested *C. glabrata* sp. do not [108, 109].

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 mitogen-activated protein kinase Mkc1p, a key component of the signal transduction pathway triggered by cell wall stress [110]. *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 Ca2+-calmodulin-

activated serine/threonine-specific protein phosphatase important for homeostasis, morphogenesis, and virulence [111]. 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 Hsp90p, a heat shock protein responsible for stabilization of a variety of host proteins [112]. In planktonic conditions, Hsp90p associates with both calcineurin and Mck1p. Although expected to govern *Candida* biofilm azole resistance through a similar pathway, disruption of Hsp90 did not impact these proteins during biofilm growth [112]. Therefore, there appears to be a distinct stress pathway promoting *C. albicans* biofilm resistance that involves Hsp90p. The finding that disruption of this pathway also impairs matrix glucan production suggests the possibility that Hsp90p may act as a regulator of the matrix sequestration pathway [112].

Conclusion

Upon transitioning to the biofilm lifestyle, *Candida* becomes extraordinarily resistant to anti-infectives and host defenses. The process that *Candida* spp. undergo to withstand conventional antifungals is multifactorial and varies 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 [72]. 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 [100]. The activation of stress-induced pathways, including the calcineurin, Hsp90, and mitogen-activated pathways, contributes to *Candida* biofilm resistance. Mature biofilms often develop persister cells, phenotypic variants that are resistant to antifungals and provide an inoculum for regrowth after drug exposure [105]. 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 Perspectives

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 which 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 [89-92, 95]. Both carbohydrate and extracellular DNA components have been linked to multi-drug resistance [90, 92, 95, 99, 100]. 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 development of novel strategies to inhibit *Candida* biofilms [16, 88, 89]. 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 [88, 89]. 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 [83, 89]. 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 (ROS) within the biofilm may be connected. Investigations of azole-treated *C. albicans* biofilms found that the persister cell population produced increased ROS compared to the rest of the biofilm [113, 114]. 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 [113]. 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 shown clinically to select for *Candida* strains with relatively larger populations of persister cells [109]. 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, but holds many unanswered questions. 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 [115]. 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 [86, 116, 117]. Promising results have been published on inhibiting bacterial biofilms by targeting their quorum sensing mechanisms [118]. In *C. albicans*, exploitation of farnesol has been proposed for this purpose [71]. However,

this molecule has not been definitively linked to drug resistance and has the potential to promote dissemination of infection to distant sites [83, 119, 120]. 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 in central venous catheter infections, particularly in cases where the catheter cannot be removed [96, 121, 122]. Liposomal amphotericin B has been shown to have activity against *Candida* biofilms in vitro and in animal models when used as an antifungal lock therapy as well [96, 122, 123], 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 [10, 11, 63, 124]. Like the beta-lactamase inhibitors used to overcome beta-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 [100, 125-132]. 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 [111]. Other drugs targeting stress responses or other biofilm-specific mechanisms of resistance may also improve the activity of conventional antifungals, eradicating these resistant infections.

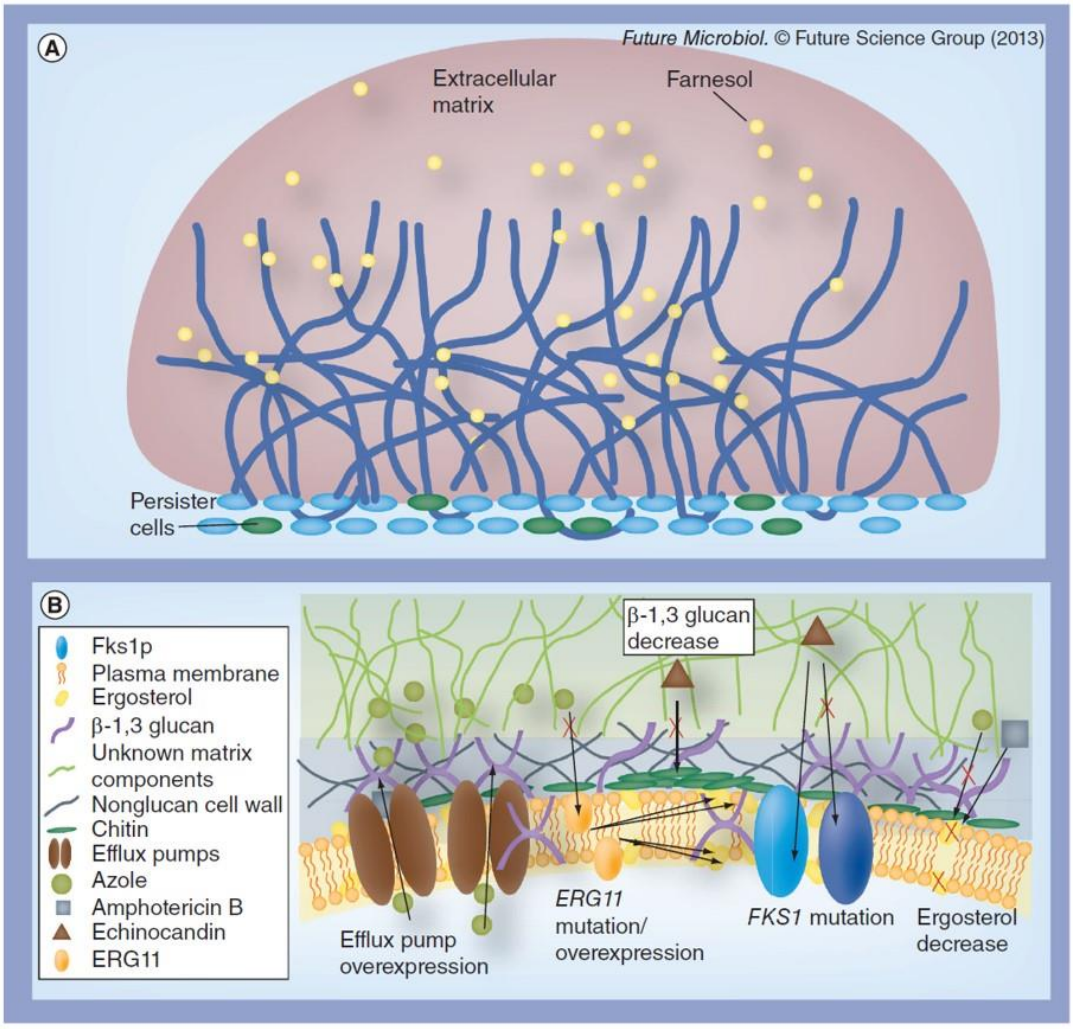

Figure 1. *Candida albicans* biofilm resistance mechanisms. Resistance mechanisms at the (A) community and (B) cellular level.

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Chapter 1: Literature Review

Section 3: The Extracellular Matrix of Fungal Biofilms

This section is in press for publication as a book chapter:

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Abstract

A key feature of biofilms is their production of an extracellular matrix. This material covers the biofilm cells, providing a protective barrier to the surrounding environment. During an infection setting, this can include such offenses as cells and products of the immune system as well as drugs used for treatment. Studies over the past two decades have revealed the matrix from different biofilm species to be as diverse as the microbes themselves. This chapter will review the composition and roles of matrix from fungal biofilms, with primary focus on *Candida* species, *Saccharomyces cerevisiae, Aspergillus fumigatus*, and *Cryptococcus neoformans*. Additional coverage will be provided on the antifungal resistance proffered by the *Candida albicans* matrix, which has been studied in the most depth. A brief section on the matrix produced by bacterial biofilms will be provided for comparison. Current tools for studying the matrix will also be discussed, as well as suggestions for areas of future study in this field.

Keywords

Extracellular matrix, biofilm, antifungal resistance, *Candida albicans*, device-associated infections

Introduction

A defining feature of the biofilm lifestyle is production of an extracellular matrix. The matrix surrounds the cells within the biofilm, providing a structural scaffold for both adhesion to surfaces and cohesion between cells [1, 2]. Additionally, the matrix of certain organisms can contribute to the retention of water and nutrients, and in some cases these nutrients are thought to derive from matrix materials hydrolyzed by microbial-produced enzymes [1]. Perhaps the most medically-relevant function of the extracellular matrix is its ability to provide a physical barrier between biofilm cells and the surrounding environment. In a human host, this includes cells and products of the immune system and often drugs used for treatment [3, 4].

The composition of the matrix has been comprehensively studied in only a handful of biofilm species. Most of these have occurred in bacteria, though the fungi *Saccharomyces cerevisiae, Aspergillus fumigatus*, and *Candida albicans* serve as exceptions. The diversity of biochemical entities discovered, and the observation that matrix components can be similar to components of the fungal cell wall, suggests that the composition of biofilm matrices can be as diverse as the microbes that produce them [5, 6]. This chapter will review the composition and roles of matrix from fungal biofilms, with a brief coverage on bacterial matrices for comparison. Current tools for studying the matrix will also be discussed, as well as suggestions for areas of future study in this field.

Matrix of Bacterial Biofilms

Most studies of biofilm extracellular matrix components to date have utilized bacterial species. One well-characterized model system involves the gram-positive, spore-forming bacterium *Bacillus subtilis*, which can form pellicles or submerged biofilms depending on the strain and environmental conditions [7]. Several polymeric components of the *B. subtilis* matrix have been described, such as poly-DL-glutamic acid (PGA) and the proteins TapA, TasA, and BslA. This matrix also contains exopolysaccharide (EPS), which is produced by the *eps* operon [8]. Notably, the EPS component was recently discovered to be at least partially composed of poly-N-acetyl glucosamine (PNAG), a widely conserved bacterial polysaccharide [9], and had previously been shown to act as a positive regulator of its own synthesis [10].

A second model organism for the study of biofilm formation is *Pseudomonas aeruginosa*, which produces an extracellular matrix containing polysaccharides, extracellular DNA (eDNA), lipids (namely rhamnolipids), and proteins [11]. Polysaccharides include Psl, Pel, levan, and alginate, whose quantity depends on the strain in question, the stage of biofilm development, and the site of biofilm formation [12]. For example, strains designated mucoid or non-mucoid differ in their overexpression of alginate, which occurs during infections of the lungs of cystic fibrosis

patients [11, 13]. The other polysaccharides are typically produced by environmental strains or those isolated from other types of infections. Pel has been extensively studied, and found to lend a protective role against aminoglycoside antibiotics [14]. DNA in the *Pseudomonas* matrix also contributes to structural stability, and has been found to induce antibiotic resistance [15, 16].

Other molecules have been identified in the matrices of multiple bacterial biofilms. Namely, cellulose is a critical component for *Escherichia coli*, *Klebsiella pneumonia*, *Enterobacter* spp., *Citrobacter* spp., and *Salmonella typhimurium* [5, 17, 18]. PNAG, identified in *B. subtilis*, is also found in *Staphylococcus aureus* and *Staphylococcus epidermidis*, as well as the related molecule polysaccharide intercellular adhesin (PIA) [5].

In addition to the composition and production of individual matrix components, assembly of the entire matrix over the course of bacterial biofilm development has been explored in two species. The first of these is *Vibrio cholerae*, which produces a matrix of *Vibrio* polysaccharide (VPS) and the proteins RbmA, RbmC, and Bap1 [19-21]. A 2012 study by Berk et. al. revealed distinct stages of cellular clustering during biofilm formation, with RbmA providing initial adhesion between cells and Bap1 aiding in surface adhesion. Cell clusters were surrounded by mixtures of VPS, RbmC, and Bap1 in the later stages of biofilm development [22]. Similarly, “spatially segregated” subpopulations of cells surrounded by extracellular matrix were observed within *E. coli* pellicles in a 2013 publication by Hung et al. In addition, this study confirmed that amyloid fibers formed by curli as a major matrix constituent, with cellulose, flagella, and type 1 pili also involved [18]. Together, these recent findings support the notion that biofilm matrix is not simply a disorganized conglomerate, but rather specific microenvironments of components that emerge in time and space.

Fungal matrix

Multiple types of fungi form biofilms, found both in the environment and in infection settings. The first investigations of the extracellular matrix of fungal biofilm species were

performed by the Douglas group, with *Candida albicans*, nearly two decades ago [23, 24]. Since that point, several groups have investigated basic aspects of matrix production and its individual components. However, not until the past few years has a larger picture of the matrix emerged, using multiple approaches to examine how its various entities collectively function to provide structural integrity and protection to the cells of biofilms.

Tools for studying the matrix

It has been proposed that successful quantification of the entire extracellular matrix is nearly impossible, as matrix isolation methods could potentially disrupt the cell wall [1]. However, recent protocols have demonstrated no change in the chemical composition of the cell wall following matrix extraction. These methods have used either mild detergents (or none at all), combined with gentle sonication to separate the cells from the matrix material [6, 25, 26].

Biochemical approaches have been used to analyze the different classes of macromolecules found in extracellular matrices. To quantify the gross amount of protein, colorimetric assays such as bicinchoninic acid (BCA), Bradford, Folin-Lowry, Kjeldahl or ultraviolet absorption can be employed. For identification of specific proteins, chromatographic and electrophoretic separation strategies have been extensively used followed by various mass spectrometry (MS) analytical techniques [6, 25]. Recent advances in bottom-up proteomics technologies have been especially important to the study of extracellular matrix, as they allow for in-depth relative and absolute quantification of proteins in intact or minimally-processed biological samples [27]. For example, our group recently utilized shotgun proteomics to achieve global protein identification in the *C. albicans* biofilm matrix [6].

Overall measurement of monosaccharides can be accomplished using the phenol-sulfuric assay [28]. A specific assay for β-1,3 glucan quantification is the limulus assay, which has been used both in vitro and as a diagnostic in clinical settings [29, 30]. For the most precise quantification of carbohydrates gas chromatography is the gold standard, and is typically coupled

with MS analysis for qualitative purposes. For structural analysis, complementary approaches utilizing GC, MS, and multiple nuclear magnetic resonance (NMR) procedures provide the granularity needed for accurate structural assignments. Similar to techniques previously used to analyze the *C. albicans* cell wall, a combination of several solution-state ^{1}H and ^{13}C NMR techniques have been used to examine the matrix.[6, 31]. Recently, solid-state ^{13}C, ^{15}N, ^{31}P NMR spectroscopy has also proven a powerful ally in generating more complete descriptions of macromolecular assemblies [25, 32]. For more detailed information on the molecular size and shape of individual matrix polysaccharides, small-angle X-ray scattering (SAXS) has also been employed.

Lipids in the extracellular matrix have also been quantified using gas chromatography. For crude separation of different lipids, TLC can be used, though the most detailed data can be achieved using MS-based shotgun lipidomics. The system recently employed by our group involved liquid chromatography (LC)/mass selective detector (MSD) time of flight (TOF) with electrospray ionization [6]. This technique has also been employed for analysis of small molecular weight lipophilic molecules, such as ergosterol and other sterols.

The final class of macromolecule, nucleic acids, can be measured in crude matrix material spectrophotometrically or with use of specific dyes [6, 33]. In our group's recent study, the potential presence of coding regions was examined by creating a clone micro-library of random regions of matrix DNA, followed by sequence homology analysis to the Candida Genome Database.

For measurement of known matrix entities, monoclonal antibodies have been produced for both imaging purposes and quantification in microtiter based assays such as ELISA [6, 34]. For further visualization of the matrix material, both SEM and TEM have been used, as well as atomic force microscopy (AFM) [15, 35].

Saccharomyces cerevisiae

The yeast *Saccharomyces cerevisiae* has long been used as a model organism for many basic aspects of eukaryotic biology, and as a means to investigate fungal biology in a non-pathogenic system. In the last fifteen years, it has also been explored as a model for the biofilm lifestyle. Certain strains of *S. cerevisiae* exhibit flocculation, or clumping of cells, and others can form true surface-adherent biofilms [36, 37]. Both flocculation and biofilm formation rely on the FLO family of cell surface adhesins, or flocculins, which are related to the ALS family and the Hwp1p adhesins in *Candida albicans* [38-40]. Of these, FLO11 is required for *S. cerevisiae* biofilm formation [39-41].

S. cerevisiae can produce extracellular matrix in both the flocculating and biofilm forms, as well as in structured colonies formed by environmental isolates. This matrix material has been visualized using electron microscopy [42-45]. The 2009 study by Beauvais et al. examined the composition and role of the matrix in FLO1-expressing cells, which exhibit strong flocculation and higher resistance to stress and drugs [45]. Flo1p aids in flocculation by interacting with sugars on the cell walls of neighboring yeast, as its exposed N-terminus possesses lectin-like properties [46, 47]. Matrix from the ‘floc’ was extracted using EDTA and shown to be loosely attached to the cell surface. The material contained mainly glucose and mannose, with a negligible amount of protein. GC-MS analysis revealed the mannose portion to be a chain of (1-6) mannan with (1-2) and (1-3) linked branches. Beauvais et al also showed the matrix from flocculated *S. cerevisiae* was able to exclude high molecular weight molecules such as antibodies or concanavalin A. However, smaller entities, namely amphotericin B and ethanol, were not blocked in this case.

Additional studies have described components of the *S. cerevisiae* matrix produced under different growth conditions. A study with environmental isolates formed ‘fluffy’ colonies, as opposed to the smooth surface produced by laboratory strains. Matrix from the environmental strains contained an unidentified protein unrelated to flocculins. The matrix material also reacted with concanavalin A, indicating the presence of exposed terminal mannose or glucose residues

[43]. More recent investigations involved cells grown in a three-dimensional mat on solid medium, and used multiple analytical techniques to identify protein and carbohydrate matrix components in both *S. cerevisiae* and *C. albicans* [48, 49]. A number of proteins were identified with 2-DE and MALDI-TOF MS, including the dehydrogenase Tdh3p for both species. For *S. cerevisiae*, two different molecular weight polysaccharides were identified, which contained glucose, mannose, and smaller amounts of galactose. The different matrix components identified in each of these studies, especially the relative abundance of proteins between flocculating and mat forms, highlights the impact of growth conditions on the production and content of extracellular matrix. This, in addition to the methods used for analysis, must be carefully considered when comparing information from different investigations.

Aspergillus fumigatus

Aspergillus biofilms can form during chronic lung infections, such as aspergilloma, and more rarely form on medical devices in humans [50, 51]. Extracellular matrix has been found to surround these three-dimensional hyphal structures during both in vitro and in vivo in studies with *A. fumigatus*, the most common species of *Aspergillus*, with thick material covering the surface of the cells [51-53]. Extracellular matrix levels increase during the maturation of *Aspergillus* biofilms, which form after adhesion of conidia to a substrate [52, 54].

The initial study of *Aspergillus* biofilm matrix content utilized biofilms grown under aerial-static conditions in glucose yeast extract (GYE) medium [52]. The matrix was found to contain galactomannan, α-1,3 glucan, proteins, polyols, and melanin. This study, using mainly immunoassays, revealed the presence of galactomannan in both the cell wall and matrix, while α-1,3 glucan was primarily located extracellularly near the surface of hyphae. The protein content, which represented 2% of the total matrix, included three major secreted antigens: dipeptidylpeptidase V (DPPV), catalase B (CatB), and ribotoxin (ASPF1). A group of hydrophobin proteins was also identified, with purported roles in intercellular adhesion during aerial growth.

Additionally, eDNA that matches genomic DNA sequence has been identified in the *A. fumigatus* biofilm matrix [55]. Interestingly, host DNA may also play a role in the *Aspergillus* matrix, as addition of exogenous DNA resulted in greater structural integrity and matrix carbohydrate production. This could reflect the conditions of the cystic fibrosis (CF) lung, where concentrations of human DNA are relatively high, and provides an interesting glimpse into the potential role of host factors in the matrix of biofilm infections.

A more recent study grew *A. fumigatus* biofilms in RPMI 1640 medium and utilized solid-state NMR to define matrix composition [25]. Multiple approaches were combined to examine facets of the entire matrix: ^{13}C, ^{15}N, and ^{31}P-NMR, electron microscopy, and protein identification using PAGE and mass spectrometry. Overall, this investigation found the matrix to contain roughly 40% protein, 43% polysaccharides, up to 14% lipids, and 3% aromatic-containing components such as melanin. Combined with existing studies that have identified specific matrix components, work such as this provides powerful quantitative data to frame future investigations of matrix structure and function.

In vivo *Aspergillus* biofilm matrix has also been investigated, using both aspergillomas from human patients and a murine model of invasive pulmonary aspergillosis [51]. Similarly to in vitro *A. fumigatus* biofilms, both biofilm models contained galactomannan within the cell wall and matrix. One observed difference between the in vivo models was the lack of matrix α-1,3 glucan in the pulmonary aspergillosis biofilms. However, they both differed from the in vitro model in their relatively higher levels of galactosaminogalactan (GAG), a cell wall exopolysaccharide that has been characterized by the Sheppard group [56, 57]. GAG has been found to mediate adherence, and plays a role in the host immune response partially by masking β-1,3 glucan and also through inducing interleukin-1 receptor antagonist (IL-1Ra), which blocks proinflammatory IL-1 signaling [58, 59].

Like the *C. albicans* matrix, the matrix of *Aspergillus* biofilms is thought to contribute to antifungal resistance, either through slowing drug diffusion or perhaps through a more specific

sequestration mechanism [52, 55, 60]. One matrix material that has been identified to have a role in *A. fumigatus* biofilm resistance is eDNA [55, 61]. An in vitro study by Rajendran et al. showed that the eDNA, which accumulates over biofilm maturation, can be degraded by DNase to result in higher biofilm susceptibility to amphotericin B and caspofungin. A possible mechanism by which eDNA enters the matrix is autolysis, as chitinase activity was found to increase DNA release. An additional study yielded increased *A. fumigatus* biofilm susceptibility to amphotericin B after treatment with alginate lyase, which degrades uronic acid-containing carbohydrates [60]. One explanation for this result is that a currently unknown carbohydrate is also contributing to antifungal resistance in the *Aspergillus* matrix.

Cryptococcus neoformans

Cryptococcus neoformans, which can cause meningoencephalitis in humans, has the ability to form biofilms on polystyrene plates and on the surfaces of medical devices such as the shunts used to treat intracranial hypertension [34]. During biofilm formation, extracellular matrix increases over time and provides structure and complexity to groups of adherent cells [62, 63].

This basidiomycete typically grows in a yeast-like form covered by a polysaccharide capsule composed of glucuronoxylomannan (GXM), a structure required for virulence. GXM is shed into the environment, and the capsule structure is required for biofilm formation [64]. Initial studies of the *C. neoformans* biofilm matrix sought to identify GXM using monoclonal antibodies. This confirmed the presence of GXM surrounding the cells of the biofilm, and GC/MS analysis detected monosaccharide content consistent with the presence of GXM. However, this also detected monosaccharides not found in GXM (glucose, ribose, and fucose) suggesting the matrix contains additional types of polysaccharides [63].

The *Cryptococcus* matrix is thought to protect the biofilm cells from surrounding stressors in both an infection setting and in the environment, where the fungus is associated with pigeon excreta [63, 65, 66]. *C. neoformans* biofilms are more resistant to changes in temperature, pH,

and UV light compared to planktonic cells, lending evidence to the idea that biofilm growth in nature provides a protective advantage [63]. Similar to biofilms formed by other infectious fungi, those of *C. neoformans* display higher levels of antifungal resistance compared to planktonically-grown cells [65], and are also more resistant to antimicrobial molecules produced by cells of the innate immune system [67]. Interestingly, a study with both *C. neoformans* and *C. gattii* showed the formation of biofilm-like microcolonies to be associated with successful resistance to phagocytosis, as well as escape from macrophages. These microcolonies were surrounded by a polysaccharide matrix containing antibody, suggesting utilization of antibody-mediated agglutination in favor of the fungus [66].

Candida

Candida albicans is the most common fungal pathogen, and frequently forms biofilms on implanted medical devices. The first studies describing the *C. albicans* biofilm matrix were conducted by the Douglas group, and demonstrated the importance of environmental conditions on overall matrix production [24]. Conditions of continuous flow, which most closely resemble those of in vivo biofilm growth, resulted in biofilms with the most abundant matrix. In support of this, in vivo biofilms from both the catheters of patients as well as multiple animal models produce visibly ample matrix [68-71].

The content of the *C. albicans* matrix initially identifed carbohydrates, hexosamine, phosphorus, protein, uronic acid, and eDNA [72, 73]. Subsequent studies identified β-1,3 glucan as one specific carbohydrate component [29, 30]. Several groups have also investigated variations in matrix content between other *Candida* species, and have noted differences in overall carbohydrate and protein content [73, 74]. Notably, the major matrix component identified in *C. tropicalis* was hexosamine, and biofilms of this species were also disrupted by different enzymatic treatments than those of *C. albicans.*

A comprehensive analysis of the *C. albicans* matrix was recently performed by our group [6]. All four classes of macromolecules were analyzed, and by dry weight was comprised of 55% protein, 25% carbohydrate, 15% lipid, and 5% nucleic acid. The relative abundance of protein was much greater than previously reported [73], and proteomic analysis indicated 458 distinct entities. This revealed many similar protein classes to those previously identified in other studies, including those implicated in carbohydrate and amino acid metabolism [48, 75]. Lipids in the *C. albicans* matrix include both neutral and polar glycerolipids, and a small proportion of sphingolipids. The nucleic acids identified were mainly non-coding sequences of DNA.

Our study included a detailed analysis of the matrix polysaccharides. Surprisingly, a previously identified carbohydrate with functional roles in the matrix, β-1,3 glucan, was found in much smaller quantities compared to two other polysaccharides, β-1,6 glucan and α-1,6 mannan with α-1,2 linked branches. While these have some similarity to the polysaccharides found in the *C. albicans* cell wall, several lines of evidence suggest that matrix and cell wall carbohydrates are produced or assembled in a distinct manner [76]. First, the three polysaccharides were found to physically interact in a mannan-glucan complex (MGCx), a structure not described in the cell wall. Second, the cell wall and matrix polysaccharides are found in different proportions to one another and with different linkages. For example, the length of mannan chains in the matrix can reach up to 12,000 mannose residues, whereas those in the cell wall have only been reported up to 200 residues. Finally, the cell wall polysaccharide chitin was not detected in the extracellular matrix, providing further evidence that cell wall and matrix are distinct.

Subsequent investigations defined the genetic basis for each of these matrix polysaccharides by screening a library of mutant strains lacking enzymes in each of the carbohydrate production pathways [77]. A subset of mutants was found to have lower levels of total matrix, lower quantities of each polysaccharide, as well as increased susceptibility to antifungal treatment (detailed below). Seven genes were identified that govern levels of matrix mannan (*ALG11, MNN9, VAN1, MNN4-4, PMR1,* and *VRG4*), while two genes govern levels of

matrix β-1,6 glucan (*BIG1* and *KRE5*). The previously studied gene encoding the β-1,3 glucan synthase, *FKS1*, was included in order to study this third matrix polysaccharide. Strikingly, when we grew mixed biofilms containing mutants from the different pathways, matrix of normal structure and function was restored. This suggested that the MGCx components assemble extracellularly, as the mutants lacking one polysaccharide could be complemented by neighboring cells lacking a different polysaccharide. These findings are represented in **Figure 1**. Reflecting a community behavior, the assembly of matrix materials by the biofilm constitutes an exciting area for further studies.

Role of *Candida* matrix in drug resistance

The early biofilm studies by the Douglas group began to explore the role of *Candida* biofilm matrix in resistance to antifungal therapies. The growth conditions of continuous flow, as mentioned above, provided the highest level of protection from drug treatment [72, 73]. It should be noted that antifungal resistance in *C. albicans* is multifactorial, with the matrix emerging as a relevant mechanism during the mature stage of biofilm growth [78, 79]. Namely, efflux pumps reduce intracellular accumulation of triazole antifungals during the early phases of biofilm growth [80, 81]. Additional mechanisms thought to impact biofilm drug resistance include the presence of drug-tolerant persister cells and changes in the sterol content of the cell membrane [80, 82, 83].

Matrix contribution to biofilm resistance was tested even more directly in a 2007 study from our group [29]. Extracellular matrix was isolated from biofilms and added to planktonic cells prior to antifungal susceptibility testing. The planktonic cells with matrix added were found to gain levels of resistance similar to mature biofilms. Further, an additional experiment tracked the penetration of radiolabeled fluconazole within biofilms, and found the majority to be retained within the matrix. This work also first implicated the matrix carbohydrate β-1,3 glucan in the resistance phenotype: biofilms treated with β-1,3 glucanase greatly increased susceptibility to fluconazole

treatment both in vitro and in vivo. A 2010 study by the d'Enfert group corroborated the importance of β-1,3 glucan by demonstrating direct binding of amphotericin B to biofilms and β-glucans [84]. Further, β-1,3 glucan plays a role in the resistance against additional classes of antifungals, and in the species *C. glabrata, C. parapsilosis,* and *C. tropicalis* [78, 85-87].

Several studies have investigated the production and regulation of β-1,3 glucan in the matrix. The β-1,3 glucan synthase Fks1p is clearly involved directly in the levels of this matrix material, and the glucan modifier proteins Bgl2p, Phr1p, and Xog1p were found to impact delivery of β-1,3 glucan from the cell surface to the extracellular space [85, 88, 89]. A separate regulatory mechanism for β-1,3 glucan involves the transcription factor Zap1p, which negatively impacts the production of this material [90]. Nobile et al found relevant targets of Zap1p to be two glucoamylases, *GCA*1 and *GCA*2, which most likely contribute to the hydrolysis of matrix carbohydrates. Other targets include alcohol dehydrogenases ADH5, CSH1, and LFD6, which could perhaps impact the biofilm and matrix through quorum sensing pathways. The most recent work from our group found that β-1,3 glucan, while present in relatively low quantities, works in conjunction with the other matrix polysaccharides to provide the biofilm resistance phenotype [77].

Several other pathways have been implicated in the resistance of *C. albicans* biofilms. The transcription factor Bcr1p, a regulator of overall biofilm formation, has been found to influence permeability to dyes and neutrophils, as well as sensitivity to fluconazole. This was observed through naturally different expression levels of Bcr1p in mating competent and mating incompetent biofilms, as well as the finding that Bcr1p was required for abundant matrix levels in a vaginal colonization model [91, 92]. The heat shock protein Hsp90p is also a putative regulator of matrix production, and therefore drug resistance, as disrupting this pathway results in azole susceptibility and also lower levels of β-1,3 glucan in the matrix [93]. Similar phenotypes were yielded by disrupting either *SMI*1 or *RLM*1, members of the protein kinase C (PKC) pathway [94]. These defects were restored by overexpression of *FKS*1 in the mutants, though no effect was seen when upstream components of the PKC pathway were disrupted. Thus, the pathway

members that impact drug resistance and the integrity of the cell wall are thought to overlap in a distinct manner from the whole PKC pathway.

An additional matrix component found to impact antifungal resistance is extracellular DNA (eDNA). Addition of DNase to biofilms increases the efficacy of some but not all antifungal drug classes [33, 95]. As described above, eDNA is an important structural component of other biofilm species, and in bacterial species is related to the exchange of genetic material [16, 55]. However, in *Candida* biofilms the exact mechanism of how eDNA contributes to drug resistance remains unclear, especially in light of these sequences being largely non-coding [6].

Conclusions

The studies outlined here have provided great insight into key components of the fungal biofilm extracellular matrix, as summarized in **Table 1**. However, continued elucidation of matrix composition, production, and function is critical for several reasons. Besides providing fundamental information on the biofilm lifestyle through a basic science lens, characterization of new matrix materials can constitute investigation of novel targets for antifungal therapies. Based on this information, if methods of matrix inhibition are developed, they might prove most useful in combination with existing antifungals that, alone, are ineffective against biofilms. As proof of principle, adding α-mannosidase or β-1,6 glucanase to biofilms in combination with fluconazole results in a greater reduction of biofilm growth in comparison to either treatment alone [77]. Additionally, specific matrix components are upregulated during biofilm growth in contrast to planktonically grown cells, with some present only during the biofilm lifestyle. These could certainly serve as diagnostic markers for biofilm infections of medical devices, which are often difficult to differentiate from other types of fungal infection.

To best advance our existing knowledge of the extracellular matrix, future studies should focus on several topics. First, the roles of specific matrix components should continue to be investigated as the components themselves are identified. While our image of the matrix is

becoming that of a complex structure, with individual components acting in concert, some of these components may be more critical than others in conferring certain physical properties. These queries will expand as studies involving mixed species biofilms become more prevalent: do biofilms with multiple microbes contain interacting matrix components? Do these inhibit or promote the overall integrity of that combined matrix, or perhaps lead to the alteration of cellular processes? Further, what is the role of host components that interact with the fungal matrix during biofilm infections?

The specific environmental triggers that either promote or inhibit matrix production should also be further characterized. The earliest studies of *Candida* biofilm matrix showed that continuous flow, the condition most similar to those encountered by an in vivo biofilm, were most conducive to matrix accumulation [72]. Variation in growth conditions can also drastically change the proportions of individual matrix materials, as with the case of eDNA levels from biofilms grown in different types of medium [33]. Ultimately, those conditions which best mimic the relevant infection niche should be used when possible, at least as validation for other studies. This will best inform the development of future therapeutic approaches, and will prove critical as the use of implanted medical devices and the associated biofilm infections continues to rise.

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Figure 1. Pictured is a scanning electron micrograph of a Candida albicans biofilm. The extracellular matrix is false colored in blue, red, and green, representing its three major polysaccharides. Work by Mitchell et al reveals that these components physically interact, and are each required for matrix structure and function [77].

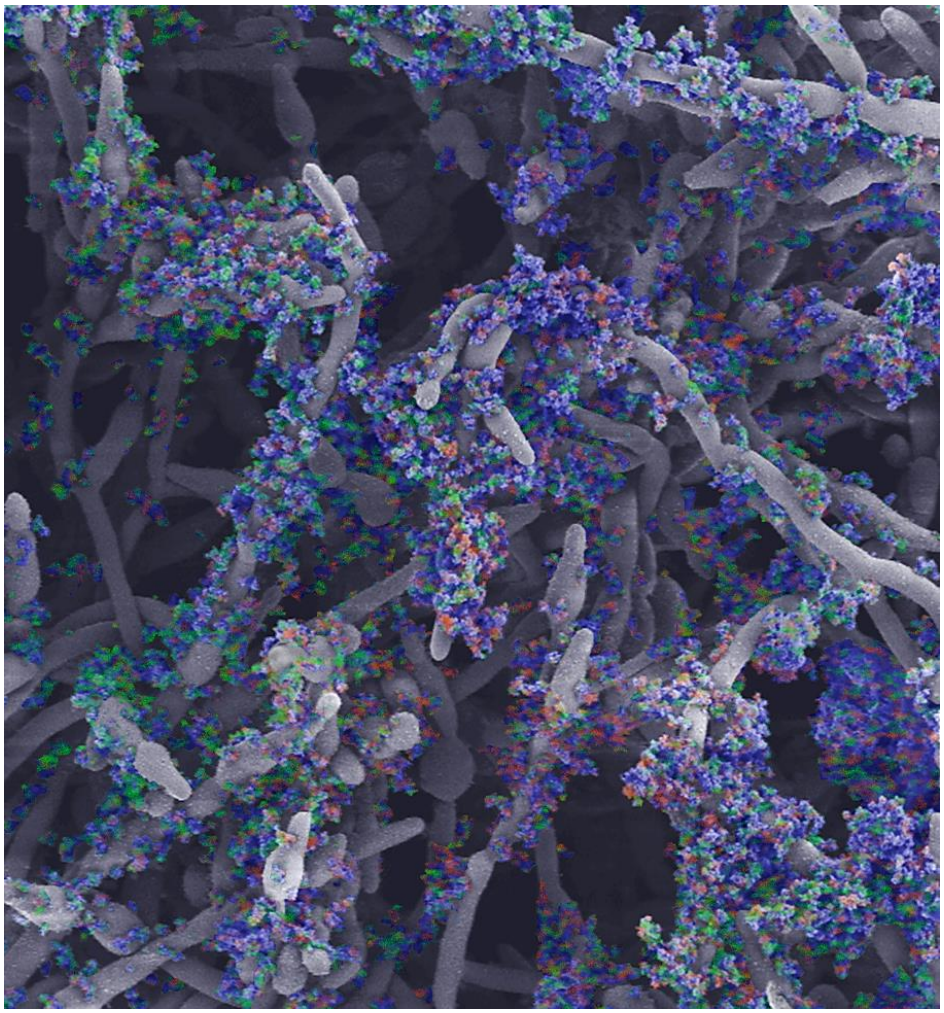

Table 1. Extracellular matrix components of fungal biofilms.

Biofilm Species	Carbohydrate	Protein	Lipid	Nucleic Acid	Other
Saccharomyces cerevisiae	Glucose, mannose in α-1,6 chains with α-1,2 and α-1,3 branches [45]; two different molecular weight polysaccharides containing glucose, mannose, and galactose [49]	Relatively low protein levels [45]; multiple proteins identified including Tdh3, Hsp26, and Sod2 [48]	--	--	--
Aspergillus fumigatus	Galactomannan, α-1,3 glucan, monosaccharides [52]; cell wall exopolysaccharide galactosaminogalactan [58]; 43% of total matrix [25]	Proteins, including hydrophobins [52]; 40% of total matrix [25]	Comprises up to 14% of total matrix [25]	eDNA [55]	Melanin, polyols [52]
Cryptococcus neoformans	Glucurunoxylomannan, xylose, mannose, glucose, galactoxylomannan [63]	--	--	--	--
Candida albicans	25% of total matrix, includes α-1,6 mannan with α-1,2 branches, β-1,6 glucan, and β-1,3 glucan [6]	55% of total matrix, with 458 distinct entities [6]	15% of total matrix, includes neutral and polar glycerolipids, sphingolipids [6]	5% of total matrix, primarily non-coding sequences [6]; eDNA [73]	Phosphorus, uronic acid, hexosamine [73]
Candida glabrata	Higher concentration of carbohydrate and protein than *C. parapsilosis* matrix [74]		--	--	--
Candida parapsilosis	Higher levels of carbohydrate than protein [74]		--	--	--
Candida tropicalis	Higher levels of carbohydrate than protein [87]; lower levels of protein than *C. albicans* matrix [73]		--	--	Phosphorus, uronic acid, hexosamine [73]

Chapter 1: Literature Review

Section 4: Extracellular Vesicles associated with *Candida albicans*

Secretion is a fundamental cellular process. One mechanism for extracellular transport of large molecules involves extracellular vesicles, or EVs. Bacterial EVs were first reported in *Escherichia coli* in the 1960s, and have since been identified in cell types across biological domains. EVs are believed to be involved in diverse functions including unconventional secretion of macromolecules, waste management, and intercellular signaling [1, 2]. In humans, they are found in blood, urine, saliva, and cerebrospinal fluid, and have been designated as markers for several types of cancer [3-6]. EVs of both endosomal and plasma membrane origin have been identified in eukaryotes, and are commonly referred to as exosomes and microvesicles, respectively [7]. In bacteria, the typical nomenclature is outer membrane vesicles (OMVs) [8]; here the abbreviation 'EV' is used to refer to these variations collectively.

Extracellular vesicles associated with fungi

EVs were first associated with *Candida,* along with several other ascomycetes, in 2008 [9]. Studies of EVs from *C. albicans* have solely used the yeast-form of the fungus. Culture supernatents were collected, concentrated, and imaged using transmission electron microscopy. Sizing analysis of these EVs suggested two populations: one ranging from 50-100 nm diameter and one ranging from 350-850 nm diameter [10]. Macrophages and dendritic cells were found to internalize *C. albicans* EVs, and macrophages have also been found to be stimulated by EVs isolated from *Cryptococcus neoformans* [11]. A few reports have suggested the presence of EVs within biofilms, but no there has been no direct evidence for the role of EVs in transport of biofilm extracellular matrix [12-15]. However, no functional testing of EVs has been performed in any species of biofilm.

Several mechanisms for the production and export of EVs have been suggested. Collectively, cellular processes including the endosomal sorting complex required for transport (ESCRT), formation of multivesicular bodies (MVBs), autophagy, and the Golgi reassembly stacking protein (GRASP; named Grh1p in *C. albicans*) have been associated with normal EV

production [17-19]. In *S. cerevisiae*, specific cellular components of both the conventional and unconventional secretory pathways were investigated [16]. This 2010 study compared EVs from wildtype yeast and several knockdown or deletion mutant strains. Mutants in the conventional secretory pathway included *SEC4* (secretory vesicle-associated Rab GTPase), *SEC1-1* (fusion of Golgi-derived exocytic vesicles with the plasma membrane), and *BOS1-1* (vesicle targeting to the Golgi). Those in other pathways were *SNF7* (involved with MVB formation) and *VPS23* (late endosomal trafficking). The involvement of multiple pathways demonstrates the complexity of the EV production process.

Cargo of *Candida albicans* yeast extracellular vesicles

Prior to the discovery of EVs, the extracellular presence of proteins that do not contain a conventional secretion signal peptide was considered an enigma. In fungi, 10-30% of the secretome does not have a predicted secretion signal [20, 21]. The protein cargo has been examined in EVs purified from *C. albicans* yeast cultures [22]. The most abundant proteins detected were *MP65* (a cell surface mannoprotein with predicted hydrolase activity), *SUN41* (a cell wall glycosidase), *SIM1* (a predicted adhesin with homology to *SUN41*), and TOS1 (a secreted protein of unknown function). Interestingly, this suggests a role for EVs in delivering polysaccharide-remodeling enzymes to the extracellular space. Other notable proteins potentially involved in this process, though less abundant in EVs, included *XOG1* (β-1,3 glucanase), *BGL2* (β-1,3 glucosyltransferase), *SUR7* (an eisosome localized protein required for normal cell wall organization), and *ENG1* (endo b-1,3 glucanase).

More recent work has characterized the nucleic acid contents of these and the EVs from several other fungi [23]. EVs isolated from *Cryptococcus neoformans*, *Paracoccidioides brasiliensis*, *C. albicans*, and *Saccharomyces cerevisiae* were all found to contain RNA molecules less than 250 nucleotides in length, which aligned with intergenic and intronic regions of mRNA, well as various ncRNAs. These findings raise the possibility of a role for EV export of nucleic acids

in interference with gene expression in host and fungal cells. The different classes of RNA identified also point to roles for EV cargo in cellular communication. DNA has not been reported in fungal EVs, but is found as EV cargo for several bacterial species including *Streptococcus mutans and Clostridium perfringens* [2].

While the possibility of carbohydrate cargo has not yet been examined in *C. albicans* EVs, this has been confirmed for other fungi. Analysis of *P. brasiliensis* and *P. lutzii* EVs yielded detection of glucose, mannose, and galactose residues, which were predicted to form two major entities: high molecular mass α-glucan and a galactofuranosylmannan [24]. *C. neoformans* EVs contain glucuronoxylomannan (GXM), which they deliver past the thick fungal cell wall to form its hallmark capsule [25, 26]. These preliminary studies suggest a variety of roles for EVs and their cargo, and could prove valuable in identifying virulence factors or markers of infection in pathogenic microbes.

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Chapter 2

Community participation in biofilm matrix assembly and function

The contents of this section were published:

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K. Mitchell performed SEM, susceptibility testing, ELISA experiments, fluconazole sequestration assays, and created new mutant strains. R. Zarnowski performed gas chromatography analyses and created new mutant strains. H. Sanchez performed the matrix co-purification study and some ELISA experiments. J. Edward and E. Reinicke performed some of the susceptibility testing and created new mutant strains.

Abstract

Biofilms of the fungus *Candida albicans* produce extracellular matrix that confers such properties as adherence and drug resistance. Our prior studies indicate that the matrix is complex, with major polysaccharide constituents α-mannan, β-1,6 glucan, and β-1,3 glucan. Here we implement genetic, biochemical, and pharmacological approaches to unravel the contributions of these three constituents to matrix structure and function. Interference with synthesis or export of any one polysaccharide constituent altered matrix concentrations of each of the other polysaccharides. Each of these was also required for matrix function, as assessed by assays for sequestration of the antifungal drug fluconazole. These results indicate that matrix biogenesis entails coordinated delivery of the individual matrix polysaccharides. To understand whether coordination occurs at the cellular level or the community level, we asked whether matrix-defective mutant strains could be coaxed to produce functional matrix through biofilm co-culture. We observed that mixed biofilms inoculated with mutants containing a disruption in each polysaccharide pathway had restored mature matrix structure, composition, and biofilm drug resistance. Our results argue that functional matrix biogenesis is coordinated extracellularly, and thus reflects the cooperative actions of the biofilm community.

Significance

Candida albicans is the most common fungal pathogen and frequently grows as a biofilm. These adherent communities tolerate extremely high concentrations of antifungals due in large part to the protective extracellular matrix. The present studies observe a novel reliance upon multiple matrix constituents for structure and function. Furthermore, the results demonstrate how the biofilm community assembles these matrix components in the extracellular space. Our findings reveal a coordinated mechanism by which the defining trait of the biofilm lifestyle arises and identify a number of potential therapeutic targets.

Introduction

In a biofilm, microbes are afforded a stable environment protected by the substrate surface and an extracellular matrix. For pathogenic microorganisms, this protection has dire consequences: it is manifested through high-level resistance to antimicrobial drugs. Hence biofilm-related infections are incredibly challenging to treat [1-5]. Elucidation of matrix biogenesis mechanisms thus addresses an interesting biological question as well as an urgent medical need.

The most common hospital-acquired fungal pathogen, *Candida albicans,* frequently forms biofilms on implanted medical devices, and often leads to lethal disseminated disease [6-8]. The intrinsic resistance of biofilms is multi-factorial, but is due largely to the extracellular matrix encasing the biofilm cells [9-19]. Although matrix accumulation is considered a biofilm-specific attribute, it has been unclear whether the matrix results from the constitutive shedding of cell wall materials or whether it reflects distinctive activities of biofilm cells. Early evidence for the constitutive secretion model came from the findings that the biofilm polysaccharide component has similar composition to the cell wall, and that the protein component of matrix is similar to the released proteins found in suspension culture [20, 21].

We have recently carried out a comprehensive analysis of *C. albicans* extracellular matrix composition [22]. We found that β-1,3 glucan, the one matrix polysaccharide that has been linked to biofilm drug resistance [23, 24], is a relatively minor matrix component. Abundant components included the polysaccharides α-mannan and β-1,6 glucan, which constituted 85% and 14% of the matrix carbohydrate fraction, respectively. Co-isolation of these components indicated that they exist in a mannan-glucan complex (abbreviated MGCx). Each MGCx component has structural features not found in the cell wall. For example, matrix mannan exists as a much larger structure (up to 12,000 mannose residues), compared to that described for cell wall mannan (approximately 150 residues) [22, 25]. Also, matrix β-1,6 glucan exists as a linear chain, while the β-1,6 glucan of the cell wall is highly branched [26]. Finally, no MGCx is evident

in cells grown in suspension culture. Therefore, the MGCx and its components' structures provide evidence for a biofilm-specific contribution to matrix biogenesis.

Our identification of matrix polysaccharide constituents provides the opportunity to dissect the mechanisms by which they yield matrix structure and protection. Here, we present evidence that mannan and β-1,6 glucan in the matrix contribute to the profound drug resistance exhibited by *C. albicans* biofilms. We also show that impaired production of any matrix polysaccharide, mannan, β-1,6 glucan, or β-1,3 glucan, diminishes deposition of other polysaccharides in the matrix. When mutant strains are combined in mixed biofilms, both matrix production and drug resistance are restored. Our findings argue that extracellular matrix is assembled extracellularly and incorporates products from a diverse biofilm community to create a unified structure.

Results

Genetic determinants of matrix polysaccharide production

In order to elucidate functions of the newly discovered matrix polysaccharides, we identified genes that govern their production. We chose 38 candidate genes that are predicted to impact the matrix mannan or β-1,6 glucan (Table S1). Six genes were chosen because of their involvement in β-1,6 glucan synthesis, and 32 genes were chosen in accordance with the mannan structure associated with MGCx. Specifically, MGCx mannan has an α-1,6 backbone with α-1,2 mannan side chains, with small amounts of phospho-linked and terminal β-1,2 linked mannose. Homozygous deletion mutants were constructed for 37 genes. After multiple attempts, the gene *MNN43* could not successfully be deleted, and therefore we used a heterozygous deletion mutant to test its function. Matrix was harvested from biofilms of each mutant and assayed for β-1,6 glucan by ELISA and mannan by gas chromatography (GC). We found that nine of the mutants had significantly lower levels of the corresponding matrix

polysaccharide than the wild-type reference strain; reductions ranged from 21-86% (Fig. 1). Our results indicate that seven genes govern levels of matrix mannan (*ALG11, MNN9, MNN11, VAN1, MNN4-4, PMR1, VRG4*), and two genes govern levels of matrix β-1,6 glucan (*BIG1, KRE5*).

Polysaccharide interactions in matrix assembly

Examination of the mutant biofilms by electron microscopy yielded a striking observation: each mutant caused nearly complete elimination of extracellular matrix (Fig. 1A). This finding is consistent with our proposal that matrix polysaccharides are in the MGCx. To test the idea that assembly of individual matrix polysaccharide components is dependent upon the others, we measured levels of all three matrix polysaccharides – β-1,6 glucan, mannan, and β-1,3 glucan – in the mutants defective in each matrix component (Fig. 1B). We used the mutant strains discussed above as well as a *TET-FKS1* strain, which has reduced β-1,3 glucan synthase expression and a consequent reduction in matrix β-1,3 glucan [27]. We also measured levels of matrix mannan and β-1,6 glucan in previously studied mutants lacking glucan modifier enzymes [28] to determine if deficient β-1,3 glucan delivery impacted matrix carbohydrate interactions (Fig. S1). Remarkably, we detected lower levels of all polysaccharides in each tested mutant strain. Because the synthesis pathways for each carbohydrate are distinct, our findings argue that there is a physical or regulatory interaction among the three matrix components.

We used pharmacological and enzymatic approaches to provide complementary evidence for the interaction of constituents in matrix assembly. Mannan accumulation was blocked with tunicamycin (TM), an antibiotic that inhibits N-glycosylation, and α-mannosidase, an enzyme that catalyzes the hydrolysis of terminal mannosides. We also examined the impact of Brefeldin A (BFA), an inhibitor of anterograde transport between the ER and the Golgi, with the goal of impairing matrix deposition of both mannan and β-1,6 glucan, as these are

transported through the secretory pathway [29, 30]. The concentrations of agents used did not inhibit planktonic or biofilm growth or alter cellular morphology (Fig. S2). As TM had previously been shown to inhibit the initial adhesion step of biofilm formation, we grew biofilms for 6 h prior to treatment [31]. Inhibition of matrix mannan synthesis by TM also reduced β-1,6 and β-1,3 glucan. This phenotype was similar, but less pronounced with α-mannosidase treatment (Fig. 1C). One explanation for our observation that matrix β-1,3 glucan levels did not decrease under α-mannosidase treatment is that the low concentrations of enzyme used resulted in incomplete hydrolysis of mannan, preventing disruption of mannan-glucan interactions. The final treatment, BFA, targeting both matrix mannan and β-1,6 glucan, also resulted in reductions in matrix β-1,3 glucan. We also performed several experiments to eliminate the possibility that biofilms with treatments inhibiting the secretory pathway did not have lower levels of matrix β-1,3 glucan due to decreased production or delivery of the glucan synthase to the cell membrane. Biofilms treated with TM or BFA had normal levels of cell wall β-1,3 glucan, indicating the synthase was able to produce normal levels of glucan (Fig. S3). Additionally, treatment of planktonic cells with TM or BFA did not decrease susceptibility to an echinocandin, indicating that the drug target Fks1p was present in comparable levels. In sum, these results support the conclusion from genetic manipulations and indicate that the three matrix polysaccharides are interdependent for extracellular accumulation in the form of biofilm matrix.

A simple model to explain polysaccharide interdependence is that all three constituents – β-1,6 glucan, mannan, and β-1,3 glucan – are physically associated in biofilm matrix. To test for such interaction, we used polysaccharide-specific monoclonal antibodies [21] in crude matrix association assays. Antibody columns were loaded with crude extracellular matrix, washed, and eluted. The eluate from each specific column contained both mannose and glucose residues (Fig. 1D and Fig. S4). This result supports a model in which biofilm matrix has physical associations between all three carbohydrates.

Several of the genes in the current study are known to have a role in the *C. albicans* cell wall [32]. Mannoproteins and β-1,6 glucan are critical components of the cell wall architecture, and are linked through a GPI-remnant [26, 33, 34]. However, none of the genes in our investigation have previously been characterized for their role in the cell wall during biofilm growth. To characterize these structures, we utilized transmission electron microscopy (TEM) to image biofilm cells. On gross appearance, the fibrillar layer of mannoproteins visible in the reference strain appeared reduced or absent in all strains with mannan defects (Fig. 2A). However, none of the mutant biofilms had significant differences in cell wall area compared to the parent strain when normalized by total cell size, suggesting the possibility of compensatory changes in individual cell wall components may allow the final structure to retain a relatively normal size (Fig. 2B). We also measured the cell wall carbohydrate composition of the mutant strains using gas chromatography as a complementary assay. The reference strain, SN250, contained 41% mannose and 54% glucose by dry weight, which is consistent with other reports for wild type planktonic cells [26]. Our results with mutant cell walls confirmed demonstrated compensatory increases in the non-mutant pathway cell wall components as has been previously described [35]. Specifically, the mannan mutant strains had higher proportions of glucose, while strains lacking a β-1,6 glucan gene had increases in cell wall mannose (Fig. 2C). We observed this general trend for all of the mutants with the exception of *mnn4-4Δ/Δ* and *van1Δ/Δ*, which maintained a proportion of mannose and glucose similar to the reference strain. The finding that all mutants had either compensatory cell wall changes or no change in their carbohydrate composition stands in stark contrast to our observations with these mutants and their extracellular matrices, where every carbohydrate component was depleted. This suggests that disruption of these carbohydrate synthesis pathways has discrete effects on the cell wall and the extracellular matrix.

Polysaccharide interactions in matrix function

We have shown previously that matrix β-1,3 glucan contributes strongly to high-level fluconazole antifungal resistance of biofilms through drug sequestration [9, 13]. Polysaccharide interaction is required for mature matrix structure, and may be required for drug resistance as well. Therefore, we assayed susceptibility of matrix mutant biofilms to fluconazole. Each of the nine mutant strains identified by the matrix polysaccharide screen exhibited a profound increase in drug susceptibility, even at the relatively low concentration of 4 μg/ml fluconazole (Fig. 3A and Fig. S5). The susceptibility phenotype was reversed in all strains in which we could introduce a wild-type copy of the deleted gene. For three mutants (*van1Δ/Δ*, *mnn11Δ/Δ*, and *kre5Δ/Δ*), we were unable to isolate transformants that carried a wild-type gene copy, but in each case multiple independent deletion mutant strains recapitulated the susceptibility phenotype (Fig. S6). The azole susceptibility of the deletion strains was specific to the biofilm mode of growth, as planktonic drug susceptibility was not altered for nearly all the mutant strains. The one exception, *alg11Δ/Δ*, was slightly more susceptible to fluconazole during planktonic growth. Mutant biofilms were also susceptible to additional antifungal classes. With the exception of one strain for amphotericin B and three strains for micafungin, greater susceptibility to these drugs and 5-flucytosine was observed in comparison to the reference strain (Fig. S7). The TET-FKS1 strain was shown previously to exhibit biofilm associated susceptibility to these antifungals [36]. Biofilm matrix of the wild-type strain was also disrupted by pharmacological and enzymatic treatments described above (TM, BFA, or α-mannosidase), and each treatment enhanced the activity of fluconazole against biofilms (Fig. 3B). We used radiolabeled drug (^{3}H-fluconazole) to determine directly if matrix mannan and β-1,6 glucan are required for drug sequestration. Compared to the reference strain, each of the mannan and glucan mutant strains had a decrease in the matrix sequestration of radiolabeled drug ranging from 10-80% (Fig. 3C). These

findings show that mannan, β-1,6 glucan, and β-1,3 glucan all contribute to matrix function as well as structure.

Extracellular assembly of matrix polysaccharide

We considered two models for matrix assembly. One possibility is that matrix is assembled extracellularly, with substrates contributed by multiple cells of the biofilm community. A second possibility is that these interactions occur intracellularly prior to export, and that biofilm properties represent only the sum total of individual cells' contributions. To distinguish between these models, we carried out an extracellular complementation assay. Specifically, we examined matrix structure and function from mixed biofilms comprised of mutant strains with defects in individual polysaccharide pathways. We reasoned that if assembly of matrix polysaccharides occurs extracellularly, then mixed mutant biofilms may produce substantial matrix to yield antifungal resistance. If assembly of matrix polysaccharides occurs intracellularly, then mixed mutant biofilms would be as defective as pure mutant biofilms. We selected one mutant strain from each carbohydrate pathway: *mnn9Δ/Δ* for mannan, *kre5Δ/Δ* for β-1,6 glucan, and *TET-FKS1* for β-1,3 glucan. All possible pairs of these mutants, as well as a triple-mixed biofilm, were assayed. We also tested mutant strains lacking glucan modifier enzymes, to explore if deficient delivery of matrix β-1,3 glucan impacts matrix carbohydrate interactions (Fig. S8). Compared to scant extracellular matrix of the individual mutant biofilms, the mixed mutant biofilms appeared similar to the reference strain by SEM imaging (Fig. 4A). Matrix carbohydrate analysis revealed that the deficiencies of each mutant strain were rescued in mixed biofilms (Fig. 4B). We also found this to be the case for mixed biofilms containing either *mnn9Δ/Δ* or *kre5Δ/Δ* mixed with a double mutant for two glucan modifier enzymes: *bgl2Δ/Δ, xog1Δ/Δ* Fig. S8A). None of the mutant strain combinations formed biofilms with significantly greater biomass than the reference strain, indicating that mixing these mutants does not confer any growth

advantage (Fig. S9A). To assay matrix function, biofilms were assayed for fluconazole susceptibility. We found that most of the mixed biofilms had similar levels of resistance to the wild-type reference strain (Fig. 4C and Fig. S8B). The one exception was the *kre5 Δ/Δ* and *TET-FKS1* mixed biofilm; its resistance was increased slightly relative to either single mutant, but we suspect that some crosslinks between β-1,6 glucan (affected by *kre5Δ/Δ*) and β-1,3 glucan (affected by *TET-FKS1*) may occur intracellularly. We verified increased radioactive fluconazole sequestration by representative mixed biofilm matrix preparations (Fig. 4D). We also tested if a mixed biofilm could restore biofilm fluconazole susceptibility in vivo, and found this to be the case for the *mnn9Δ/Δ* and *kre5Δ/Δ* combination in the clinically relevant rat central venous catheter model (Fig. 4E). These data demonstrate that mixed mutant biofilms regain the ability to produce functional matrix. Our observations indicate that, while some β-1,6 glucan – β-1,3 glucan assembly may occur intracellularly, the matrix polysaccharide components are capable of assembly after they have exited the cell.

Discussion

The extracellular matrix is one of the defining features of biofilm growth [17, 37, 38], providing a means for microorganisms to control their local environment. Matrix function is manifested clinically though the protection it affords from antimicrobial therapies. This particular function of the matrix is especially relevant in the case of *C. albicans* biofilms, which form on medical devices and exhibit tolerance of antifungals of up to one thousand times higher than those necessary to kill planktonic cells [15]. In fact, current treatment guidelines recommend removal of *Candida*-infected devices, given their persistence in the face of antifungal therapy [8, 39, 40]. Despite the biological and clinical significance of *C. albicans* biofilm matrix, we have a limited understanding of its biogenesis. Here we have used our recent analysis of matrix constituents [22] to define genetic determinants of matrix polysaccharide synthesis. We show

that each of the three major matrix polysaccharide constituents is required for assembly and function of matrix. In addition, we provide evidence that critical events in matrix assembly can occur extracellularly, and thus that matrix production represents a community activity of biofilm cells.

Two previous lines of evidence suggested that matrix biogenesis may involve a multi-component interaction. First, in our prior investigations of β-1,3 glucan, we found that genetic disruption of this single matrix component yielded biofilms with a profound lack of extracellular material, as visualized by SEM imaging [13, 28]. Second, our identification of a matrix mannan-glucan complex, MGCx, pointed to an interaction among specific matrix components that was the starting point for the current investigation [22]. Specifically, we used the composition of MGCx to develop a candidate gene list for matrix biogenesis functions. We showed that many of those candidate genes were indeed required for accumulation of their expected matrix polysaccharide product. The model that polysaccharide interaction is pivotal for matrix biogenesis predicted that matrix accumulation should be dependent upon each of the components, which was verified through SEM imaging and biochemical analysis. Moreover, we found that the protection from fluconazole afforded by matrix was also dependent upon each polysaccharide component. These results establish that matrix structure and function both depend upon multiple matrix polysaccharides.

What is the nature of the polysaccharide interaction? Given our prior identification of MGCx, and our ability to co-immunoprecipitate glucan and mannan from matrix extracts, it seems reasonable that a physical interaction among the polysaccharide components is the basis for matrix structure and function. While further structural analysis is required, we suspect these interactions could be covalent linkages, perhaps with protein intermediates. This model of physical interaction predicts that each component-specific mutant exports the other two components, but fails to assemble them into functional matrix. The results of our mixed mutant biofilms lend support for this model, as they reveal that each mutant can provide the matrix

component that is missing from a complementary mutant. Moreover, the outcome – that pathway-specific mutants participate in extracellular complementation – shows that assembly of the matrix polysaccharide complex must occur extracellularly, after export of individual components from neighboring biofilm cells. Interestingly, these mixed biofilms exhibited levels of matrix components often two-fold greater than the reference strain (Fig. 4B). While the explanation for this is unclear, a possibility is that the abnormal matrix components act in a feedback signaling process to cause an increase in mature matrix synthesis.

Our findings provide a simple explanation for the association of matrix with the biofilm growth form of *C. albicans*. The mixed mutant biofilm experiments indicate that critical steps in matrix assembly occur extracellularly, and thus that matrix assembly is a community activity. In that case, matrix assembly will be most efficient at the high cell densities that occur in a biofilm, when the substrates for assembly are at high concentrations. Viewed from this perspective, *C. albicans* matrix assembly is in essence a quorum-sensing phenomenon. The “target” of the secreted molecules that accumulate is not a surface receptor or response regulator, but instead the enzymes that catalyze MGCx synthesis and matrix assembly.

Materials and methods

Media. *Candida albicans* strains were stored at -80^{o}C in 25% (vol/vol) glycerol, and sustained on yeast extract-peptone-dextrose (YPD) medium with uridine. For mutant construction, transformants were selected on minimal medium with the corresponding auxotrophic supplements. Biofilms were grown in RPMI 1640 buffered with MOPS, and inoculated from overnight cultures grown at 30^{o}C in YPD.

Strains and strain construction. The strains developed for this study are listed in Table S1, as well as several previously reported strains that were used in the present studies. The

genotypes of strains developed in the present work are shown in Table S2. The parent strain SN152 was used to create homozygous deletion strains using fusion PCR disruption cassettes as previously reported (42). Complementation of mutant strains with a single gene copy used selection for arginine prototrophy (28). Briefly, each ORF plus 1 kb upstream and downstream was amplified by PCR, with primers designed to introduce BamHI and AscI restriction sites to the 5' and 3' ends, respectively. Cassettes were cloned into the ampicillin-resistant *E. coli* plasmid pC23, which carries the *C. dubliniensis ARG4* gene. Fusion PCR products and complementation plasmids were both transformed into *C. albicans* using the lithium acetate method. Colony PCR was used to verify all genotypes; primers are listed in Table S3.

Biofilm cell SEM. Biofilms were grown on sterile coverslips (Thermanox) in 12-well plates. 10 µl fetal calf serum was added to each coverslip and allowed to dry. 40 ul of an inoculum of 10^8 cells/ml in RPMI-MOPS was then added to the coverslips and incubated 60 min at 37^{o}C. 1 ml RPMI-MOPS with 5% fetal calf serum was added to each well and the plates were incubated at 37^{o}C for 20 h, shaking at 50 rpm. Media was removed and 1 ml fixative was added to each well (4% formaldehyde, 1% glutaraldehyde in PBS) prior to incubation at 4^{o}C overnight. The coverslips were washed with PBS, and 1% osmium tetroxide was added for 30 min incubation at room temperature. Samples were then washed with a series of increasing ethanol dilutions (30 to 100%), with 10 min incubation for each. Critical point drying was employed to completely dehydrate the samples, and they were palladium-gold coated. Samples were imaged with a SEM LEO 1530, and images were compiled using Adobe Photoshop 7.0.1.

Biofilm matrix and cell wall collection and analysis. Extracellular matrix was collected from in vitro biofilms grown in 6-well plates for 48 h, as published previously (28). Cells from an overnight culture were used to create an inoculum of 10^6 cells/ml in RPMI-MOPS. Experiments with the *TET-FKS1* mutant used RPMI-MOPS with 15 ng/ml doxycycline, and biofilm

experiments with the double mutants *bgl2Δ/Δ, xog1Δ/Δ* and *bgl2Δ/Δ, phr1Δ/Δ* used YPD as described previously (28). 1 ml inoculum was added to each well, and biofilms were incubated at 37ºC for 48 h with orbital shaking at 50 rpm, with a media exchange at 24 h. For mixed biofilm experiments, equal volumes of the inoculum for each strain were added to total 1 ml. For normalization of subsequent ELISA data, the mass of one biofilm from each group was assessed using crystal violet (Fig. S9B) (43) Biomass values for the glucan modifier strains were previously reported (28). The remaining biofilms were removed from the wells using a spatula and suspended in water, then sonicated for 20 min. These were centrifuged at 4,000 rpm for 20 min at 4ºC, separating the soluble matrix. Cell wall material was isolated from the pellet by bead beating and centrifuging as before.

For experiments with tunicamycin (1.0 µg/ml) and brefeldin A (0.6 µg/ml) (Sigma), biofilms were formed for 6 h prior to a 24 h dose. Biofilms treated with α-mannosidase (0.78 U/ml) (Jack Bean, Sigma) were formed for 24 h before a 24 h dose. Untreated biofilms were included in all assays, and crystal violet data was again used for normalization based upon biofilm cell mass.

Matrix samples were analyzed by ELISA using biotinylated-Concanavalin A (Vector Laboratories), or monoclonal antibodies to β-1,3 glucan, β-1,6 glucan, or mannan (in house) as previously described (22, 44). For measurement of β-1,3 glucan in the cell wall, samples were dissolved in DMSO and diluted to a final concentration of 4% v/v for sampling. These assays used mannan from *C. albicans* (in house), laminarin (Sigma), or pustulan (gift of Dr. Ronald Hatfield) as standards, respectively. For these assays, at least two biological replicates were performed, and the mean of three technical replicates from one representative assay was calculated. Samples were quantified using OD or luminescence on an automated plate reader. Once normalized by biomass, these values were presented as a percentage of the reference strain. Standard errors were calculated, and pairwise comparisons using ANOVA with the Holm-Sidak method were performed.

Gas chromatography analysis was used to determine the monosaccharide composition and concentration in matrix and cell wall samples. Sugars were converted to alditol acetate derivatives as previously described (45), which were detected and quantified by GLC-FID on a Shimadzu GC-2010 system. A 50% cyanopropylmethyl/50% phenylmethyl polysiloxane column was used (Restek). GLC conditions were the same as previously published (22): injector at 220 °C, detector at 240 °C, and a temperature program of 215°C for 2 min, then 4°C/min up to 230°C before holding for 11.25 min run at constant linear velocity of 33.4 cm/sec and split ratio of 50:1. Data for each monosaccharide were calculated as a percentage of the total detected sugars, and presented as a percentage of the reference strain. Standard errors were calculated, and pairwise comparisons using ANOVA with the Holm-Sidak method were performed.

Affinity purification of matrix polysaccharides. A CNBr-activated Sepharose 4B column was activated and prepared according to the manufacturer's specifications (GE Life Sciences). Monoclonal antibodies for mannan, β-1,6 glucan (22, 44), or β-1,3 glucan (Biosupplies) were resuspended in coupling buffer to a final concentration of 2.5 mg/ml, 0.8 mg/ml, or 0.3 mg/ml, respectively. The ligand and sepharose were incubated overnight while shaking at 4^{o}C. This mixture was incubated again overnight with blocking buffer, and then washed four times with high and low pH buffers (pH 8.3 and pH 4.0). The mixture was used to pack a 2 ml column, which was loaded with 10 mg/ml crude *Candida albicans* extracellular matrix (prepared as described in (22)) and washed. Elution buffer (0.2 M glycine, 0.5 M NaCl pH 3.0) was added to the column and 10-2 ml fractions were collected. These fractions were tested in the phenol-sulfuric carbohydrate assay (46) prior to analysis with gas chromatography or ELISA, as detailed above.

Biofilm cell wall TEM. Biofilms were grown in 6-well plates for 48 h as detailed above, and prepared for transmission electron microscopy as previously published (28). Cells were fixed in

4% formaldehyde and 2% glutaraldehyde, then treated with 1% osmium tetroxide and 1% potassium ferricyanide. Samples were then stained with 1% uranyl acetate and dehydrated in a series of ethanol washes. The cells were embedded in Spurr's resin, from which 70 nm sections were placed on copper grids and then poststained with 8% uranyl acetate in 50% methanol and Reynolds' lead citrate. Samples were imaged using a Philips CM 120 TEM. NIH Image J software was used to measure total cell and cell wall area of 10 cells from each strain. These values were averaged, and standard errors calculated.

Biofilm and planktonic cell susceptibility to fluconazole. The inocula for susceptibility experiments were created as described above, with 100 µl used in 96-well plates. After a 6 h biofilm formation period at 37^{o}C, media and non-adherent cells were removed. Fresh media and dilutions of fluconazole (4 to 1000 µg/ml), Amphotericin B (0.125 µg/ml), micafungin (0.03 µg/ml), or 5-flucytosine (8.0 µg/ml) were added for 48 h incubation with an additional drug and media exchange at 24 h. Biofilms treated with tunicamycin, brefeldin A, or α-mannosidase were grown following the same parameters as described above, either alone or in combination with fluconazole.

Biofilms were quantified using a tetrazolium salt XTT {2,3-bis-(2-methoxy-4-nitro-5-sulfophenyl)-2H-tetrazolium-5-carboxanilide inner salt} reduction assay (47, 48). 90 µl XTT (0.75 mg/ml) and 10 µl phenazine methosulfate (PMS) (320 µg/ml) were added for 30 min at 37^{o}C, and an automated plate reader was used to measure absorbance at 492 nm. Biofilm reduction was calculated by comparing untreated control biofilms to those with treatment. Three technical replicates from a representative biological replicate were averaged. Statistical significance was determined using ANOVA with pairwise comparisons with the Holm-Sidak method.

To assay the susceptibility of planktonically-grown *C. albicans* strains to fluconazole, the CLSI M27 A3 broth microdilution method was employed (49). For planktonic susceptibility testing to micafungin with either TM or BFA, reference cells were inoculated in 96-well round

bottom plates at 10^6 cells/ml and allowed to grow at 37°C for 6 h. 50 µl micafungin (250 to 2 ng/ml) were then added to all wells, with control groups receving 50 µl RPMI and treatment groups received 50 µl TM (1.0 µg/ml) or BFA (0.6 µg/ml) in RPMI. After 24 h incubation, the plates were spun at 3k rpm for 5 min and media was removed. Cells were quantified using XTT and PMS at the concentrations above and read at 492 nm. The reduction of cell growth was determined through comparison to untreated control wells.

In vivo testing was performed with a previously described rat central venous catheter model (50, 51). Briefly, a 10^6 cells/ml inoculum for each strain or strain combination was allowed to grow on an internal jugular catheter placed in a pathogen-free female rat for 24 h. After this period, the catheter volumes were removed and the catheters were flushed with 0.9% NaCl, prior to 24 h treatment with either 500 µg/ml fluconazole or saline. The catheters were then removed from the animals, and biofilms were dislodged by sonication and vortexing as previously described. Viable cell counts were determined by dilution plating. The treatment group for each biofilm was compared to the corresponding saline control using pairwise comparisons with ANOVA with the Holm-Sidak method.

Sequestration of ^{3}H fluconazole in biofilms. Radiolabeled fluconazole (Moravek Biochemicals; 50 µM, 0.001 mCi/ml in ethanol) was used in an adapted assay to assess drug retention in biofilms (28, 52). 6-well biofilms were grown for 48 h as described above. After washing with sterile water, biofilms were incubated with 8.48×10^5 cpm of ^{3}H fluconazole in RPMI-MOPS for 30 min at 37°C, with orbital shaking at 50 rpm. Unlabeled fluconazole (20 µM) in RPMI-MOPS was added for an additional 15 min incubation period. After washing with sterile water, biofilms were collected as detailed above. An aliquot of each collected intact biofilm was saved for scintillation counting. Extracellular matrix was isolated in the sample supernatant after vortexing as described above, and then the cells were broken by bead beating to yield cell wall and intracellular supernatant portions. Sample fractions were added to ScintiSafe 30% LSC

cocktail (Fisher Scientific) and a Tri-Carb 2100TR liquid scintillation analyzer (Packard) was used to determine counts per minute. Three technical replicates were averaged, and the standard errors were calculated. Values were compared to the reference strain using pairwise comparisons with ANOVA with the Holm-Sidak method.

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Author Contributions

The authors have made the following declarations about their contributions: K.F.M., R.Z., J.A.E., and D.R.A. designed research; K.F.M., R.Z., H.S., J.A.E., E.L.R., and D.R.A. performed research; K.F.M., R.Z., H.S., J.A.E., and E.L.R. contributed new reagents/analytic tools; K.F.M., R.Z., H.S., J.A.E., J.E.N., A.P.M., and D.R.A. analyzed data; K.F.M., J.E.N., A.P.M., and D.R.A. wrote the paper.

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Figure Legends

Fig. 1. Extracellular matrix polysaccharides interact and are required for matrix structure. (A) Biofilm morphology and extracellular matrix abundance of mutant strains and the references train SN250 (Ref) was assessed visually using SEM imaging. White arrow indicates extracellular matrix material, and scale bars represent 20 μm. (B) Carbohydrates in the extracellular matrix of biofilms were quantified using gas chromatography analysis for mannan or ELISA with monoclonal antibodies for β-1,6 glucan and β-1,3 glucan. Data are presented as percentages of the reference strain with standard errors shown. All values were significantly lower than the reference according to ANOVA ($p<0.008$). (C) Carbohydrates in the matrix of wild-type biofilms treated with tunicamycin (TM), brefeldin A (BFA), and α-mannosidase (αMS) were quantified using ELISA. Data are presented as percentages of the reference strain, with mean and standard errors shown. All values were significantly lower than the reference according to ANOVA, except the β-1,3 glucan concentration in α-MS treated biofilms ($p<0.002$). (D) Specific monoclonal antibodies for each matrix carbohydrate were conjugated to a CNBr Activated Sepharose 4B column. Purified extracellular matrix was run through each column, with each yielding one carbohydrate-positive fraction, which was analyzed using gas chromatography. The relative ratios of mannose to glucose were determined.

Fig. 2. Carbohydrate alterations in mutant biofilm cell walls are distinct from the extracellular matrix. (A) Representative images of biofilm cell wall ultrastructure, visualized using TEM. Scale bars represent 0.2 μm. (B) The area of the cell wall was measured using ImageJ software. Values were normalized by the area of the total cell, and are shown as a percentage of the reference strain. The mean and standard errors from ten individual cells are shown. (C) Cell wall

carbohydrate composition was determined using gas chromatography. The percentage of the total carbohydrates in each sample comprised of mannose and glucose is shown.

Fig. 3. Interactions of extracellular matrix carbohydrates are required for biofilm antifungal resistance. (A) The percent of reduction in biofilm formation following 48 h treatment with 1000 μg/ml fluconazole compared to untreated biofilms, as quantified using the 96-well XTT assay. The null mutant (Δ/Δ) and complemented strain (Δ/Δ + comp) are shown for each gene of interest. For *FKS1*, the *TET-FKS1* strain is shown in place of a homozygous mutant, and the heterozygote strain is shown in place of a complemented strain. The minimum inhibitory concentration (MIC) of fluconazole for planktonic cells of the Δ/Δ strains is shown below. (B) Biofilms were treated with pharmacological inhibitors or enzymes both with and without 1000 μg/ml fluconazole. Experiments used the same parameters as for the experiments in Figure 1C, but in a 96-well plate format for quantification with the XTT assay. (C) Biofilms were grown for 48 h and then exposed to ^{3}H-fluconazole. Extracellular matrix was isolated for scintillation counting, and the counts per minute (cpm) for each mutant strain were compared to the reference strain. The figure represents the mean from three technical replicates. Asterisks indicate values were significantly different than the reference strain, based upon ANOVA with pairwise comparisons using the Holm-Sidak method ($p<0.001$). Standard errors are shown for all panels.

Fig. 4. Mixed mutant biofilms have restoration of extracellular matrix structure and function. Different combinations of a mannan mutant (*mnn9*□□□), a β-1,6 glucan mutant (*kre5*□□□), and a β-1,3 glucan synthase mutant (*TET-FKS1*) were used in equal number to inoculate mixed biofilms. Assays were performed as previously described: (A) SEM, (B) matrix carbohydrate content ($p<0.05$), (C) biofilm reduction following fluconazole treatment ($p<0.005$), and (D) matrix

fluconazole sequestration ($p<0.005$). Data for single-mutant biofilms previously presented in Figure 1 are shown here for reference. Asterisks indicate the mixed mutant biofilm values are significantly different than their corresponding single mutant values. (E) The *mnn9Δ/Δ* and *kre5 Δ/Δ* mixed biofilm was tested in vivo using a rat central venous catheter model, with the effects of fluconazole or saline treatment compared to the reference and single mutant biofilms. Biofilms were quantified using viable cell counts following treatment ($p<0.006$). Statistical analyses are based on ANOVA using pairwise comparisons with the Holm-Sidak method. Mean and standard errors are shown.

Supporting Information Legends

Fig. S1. Mutants lacking β-1,3 glucan modifying enzymes have lowered concentrations of matrix mannan and β-1,6 glucan. Three single mutants and two double mutants were each grown in 6-well plates for 48 h. Extracellular matrix was collected and assayed for levels of mannan using gas chromatography and β-1,6 glucan using ELISA. All mutants had significantly lower levels of the polysaccharides compared to the reference ($p<0.01$). Values represent the mean of three technical replicates, with standard errors shown.

Fig. S2. Biofilm and planktonic cell growth following treatment with carbohydrate inhibitors. (A) Biofilms grown in 6-well plates were quantified using crystal violet. Planktonic cells were grown in 96-well round bottom plates for 24 h and quantified using XTT. For experiments with tunicamycin (TM, at 1.0 μg/ml) and Brefeldin A (BFA, at 0.6 μg/ml), biofilms or cells were grown for 6 h and then treated for 24 h before quantification. Experiments with α-mannosidase (α-MS, at 0.78 U/ml) used biofilms or cells first grown for 24 h before 24 h treatment. The mean of three technical replicates and standard errors are shown. (B) Biofilms from the above experiments were imaged using light microscopy at 25x magnification.

Fig. S3. Treatment with TM or BFA does not alter the delivery of function of the β-1,3 glucan synthase. (A) The concentration of β-1,3 glucan was measured in the cell wall of biofilms treated with either 1.0 μg/ml tunicamycin (TM) or 0.6 μg/ml brefeldin A (BFA). These values were not statistically different from the reference ($p \geq 0.270$). (B) Planktonic reference cells were grown 6 h prior to treatment with micafungin alone or in combination with either TM or BFA at the same concentrations as above. Following treatment for 24 h, remaining cells were quantified with XTT and the reduction of cell growth compared to untreated controls was determined ($p \geq 0.001$). The mean and standard error of three technical replicates are shown. Statistical analyses were based on ANOVA with pairwise comparisons using the Holm-Sidak method.

Fig. S4. Specificity of monoclonal antibodies. The antibodies used in Figure 1D (mannan, β-1,3 glucan, and β-1,6 glucan) were tested by ELISA for reactivity with laminarin, mannan, pustulan, BSA, and glucose. Values shown represent detection of an antigen concentration of 1 μg/ml using luminescence. Statistical analysis with ANOVA and pairwise comparisons indicated the detection of mannan, pustulan, and laminarin by the mannan, β-1,6 glucan, and β-1,3 glucan antibodies, respectively, was significantly greater than their detection of the other tested antigens ($p < 0.001$).

Fig. S5. Reduction of mutant biofilms following exposure to low concentrations of fluconazole. Following growth for 6 h, biofilms were treated with 4 μg/ml fluconazole for 48 h. Biofilms were quantified using the 96-well XTT assay and reduction was determined by comparing treated and untreated biofilms. All mutant biofilms were significantly more reduced than the reference strain, determined by ANOVA with pairwise comparison using the Holm-Sidak method ($p < 0.001$). The mean and standard error for three technical replicates are shown.

Fig. S6. Multiple independent transformants of non-complemented mutant strains are susceptible to fluconazole. Following growth for 6 h, biofilms were treated with 1000 μg/ml fluconazole for 48 h. Biofilms were quantified using the 96-well XTT assay and reduction was determined by comparing treated and untreated biofilms. Two independent transformants were tested for each homozygous deletion mutant, designated Transformant 1 and Transformant 2. The strains designated Transformant 1 are those used for subsequent experimentation (KMR347, ELR108, and JAE104). All mutants were significantly more susceptible to treatment than the referent strain ($p<0.001$). A different biological replicate is presented here than in Figure 2A. The mean of three technical replicates and standard errors are shown.

Fig. S7. Mutant biofilm reduction following treatment with multiple antifungal classes. Following growth for 6 h, biofilms were treated with either 0.125 μg/ml Amphotericin B, 0.03 μg/ml Micafungin, or 8.0 μg/ml 5-flucytosine for 24 h. Biofilms were quantified using the 96-well XTT assay and biofilm reduction was determined by comparing treated and untreated groups. With the exception of one strain for Amphotericin B and three for Micafungin, all strains were significantly more susceptible to treatment than the reference ($p<0.038$). The mean and standard error of three technical replicates are shown.

Fig. S8. Restoration of matrix function in mixed biofilms containing a β-1,3 glucan modifier double mutant. (A) The double mutant *bgl2Δ/Δ, xog1Δ/Δ* was grown in combination biofilms with either *mnn9Δ/Δ* or *kre5Δ/Δ* Polysaccharides were measured from the matrix using gas chromatography for mannan and ELISA with monoclonal antibodies for β-1,6 glucan and β-1,3 glucan. No measurements were significantly lower than those of the reference strain. (B) These biofilm combinations were grown in 96-well plates and treated with 1000 μg/ml fluconazole for 48 h. Bars represent the reduction in biofilm growth compared to the reference, as measured by

XTT. For both panels, ANOVA using pairwise comparisons was performed (* $p<0.001$). The mean of three technical replicates and standard errors are presented.

Fig. S9. Mixed mutant biofilm and single mutant biofilm formation. Formation of mature mixed mutant biofilms (A) and single mutant biofilms (B) were quantified using crystal violet. Biofilms were grown for 48 h in 6-well plates. The mean of three technical replicates from one representative assay and standard errors are shown.

Figure 1

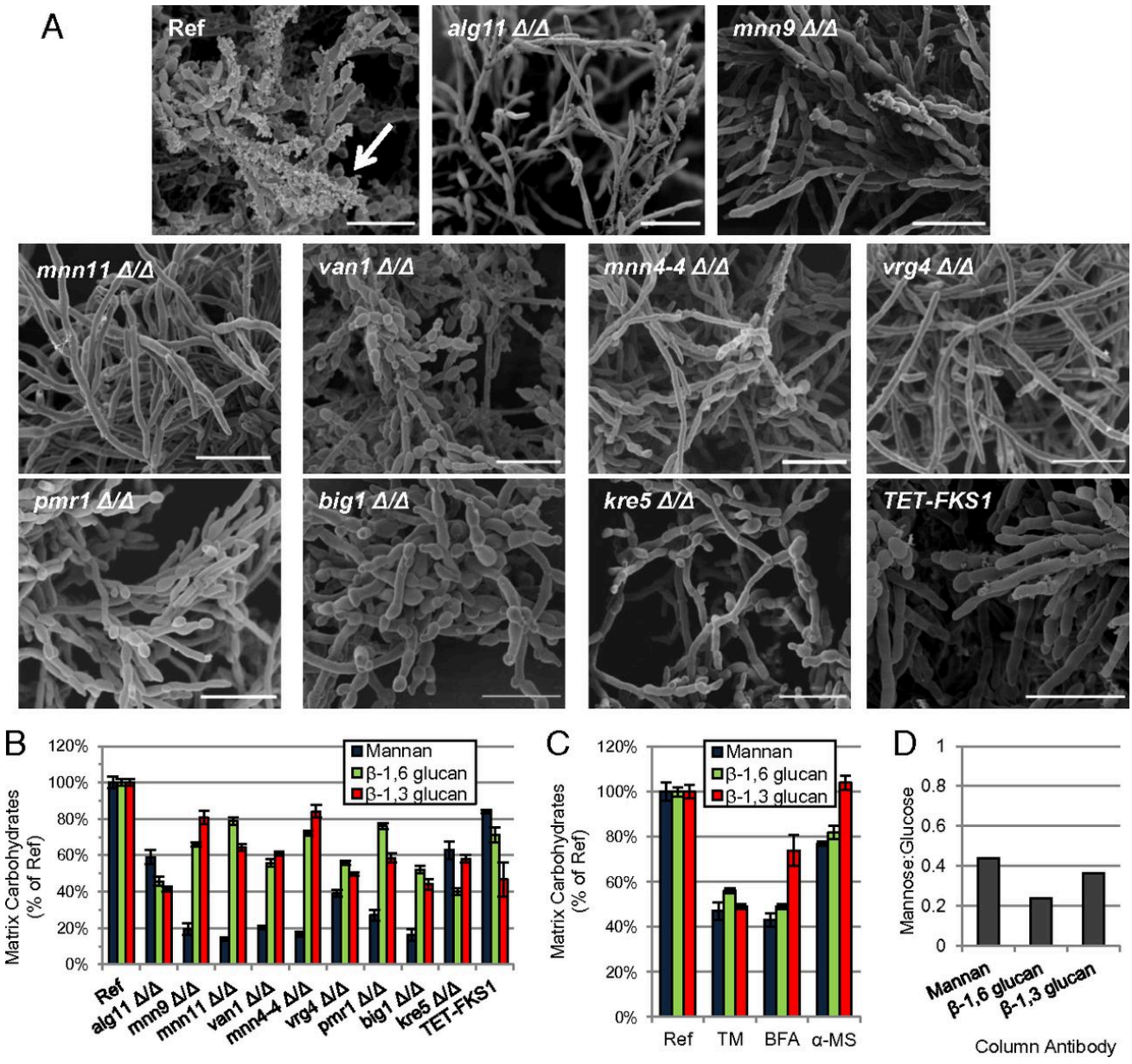

A
Ref
alg11 Δ/Δ
mnn9 Δ/Δ
mnn11 Δ/Δ
van1 Δ/Δ
mnn4-4 Δ/Δ
vrg4 Δ/Δ
pmr1 Δ/Δ
big1 Δ/Δ
kre5 Δ/Δ
TET-FKS1
B
Matrix Carbohydrates (% of Ref)
120%
100%
80%
60%
40%
20%
0%
Mannan
β-1,6 glucan
β-1,3 glucan
Ref
alg11 Δ/Δ
mnn9 Δ/Δ
mnn11 Δ/Δ
van1 Δ/Δ
mnn4-4 Δ/Δ
vrg4 Δ/Δ
pmr1 Δ/Δ
big1 Δ/Δ
kre5 Δ/Δ
TET-FKS1
C
Matrix Carbohydrates (% of Ref)
120%
100%
80%
60%
40%
20%
0%
Mannan
β-1,6 glucan
β-1,3 glucan
Ref
TM
BFA
α-MS
D
Mannose:Glucose
1
0.8
0.6
0.4
0.2
0
Mannan
β-1,6 glucan
β-1,3 glucan
Column Antibody

Figure 2

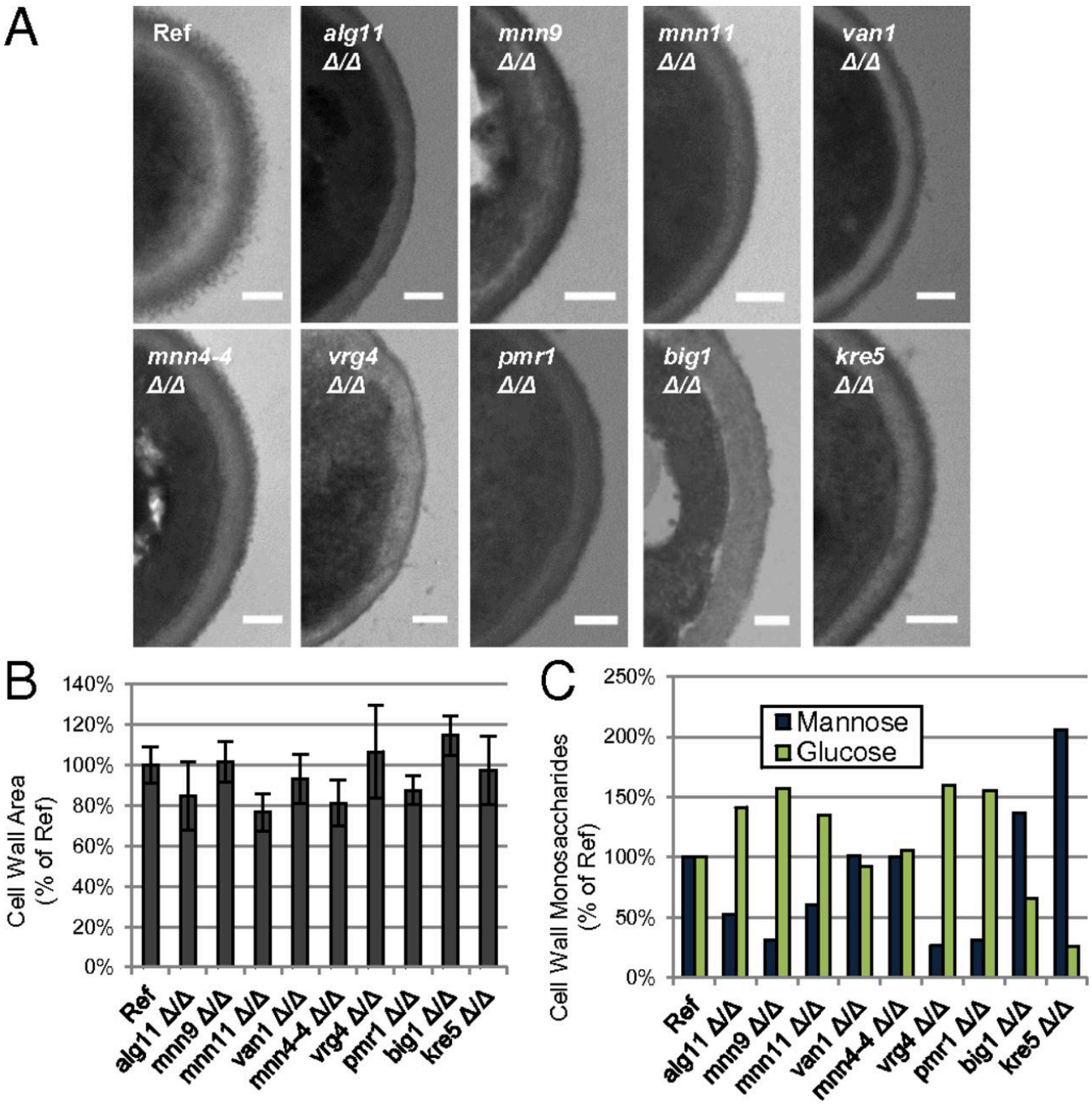

A
Ref
alg11 Δ/Δ
mnn9 Δ/Δ
mnn11 Δ/Δ
van1 Δ/Δ
mnn4-4 Δ/Δ
vrg4 Δ/Δ
pmr1 Δ/Δ
big1 Δ/Δ
kre5 Δ/Δ
B
Cell Wall Area (% of Ref)
140%
120%
100%
80%
60%
40%
20%
0%
Ref
alg11 Δ/Δ
mnn9 Δ/Δ
mnn11 Δ/Δ
van1 Δ/Δ
mnn4-4 Δ/Δ
vrg4 Δ/Δ
pmr1 Δ/Δ
big1 Δ/Δ
kre5 Δ/Δ
C
Cell Wall Monosaccharides (% of Ref)
250%
200%
150%
100%
50%
0%
Mannose
Glucose
Ref
alg11 Δ/Δ
mnn9 Δ/Δ
mnn11 Δ/Δ
van1 Δ/Δ
mnn4-4 Δ/Δ
vrg4 Δ/Δ
pmr1 Δ/Δ
big1 Δ/Δ
kre5 Δ/Δ

Figure 3

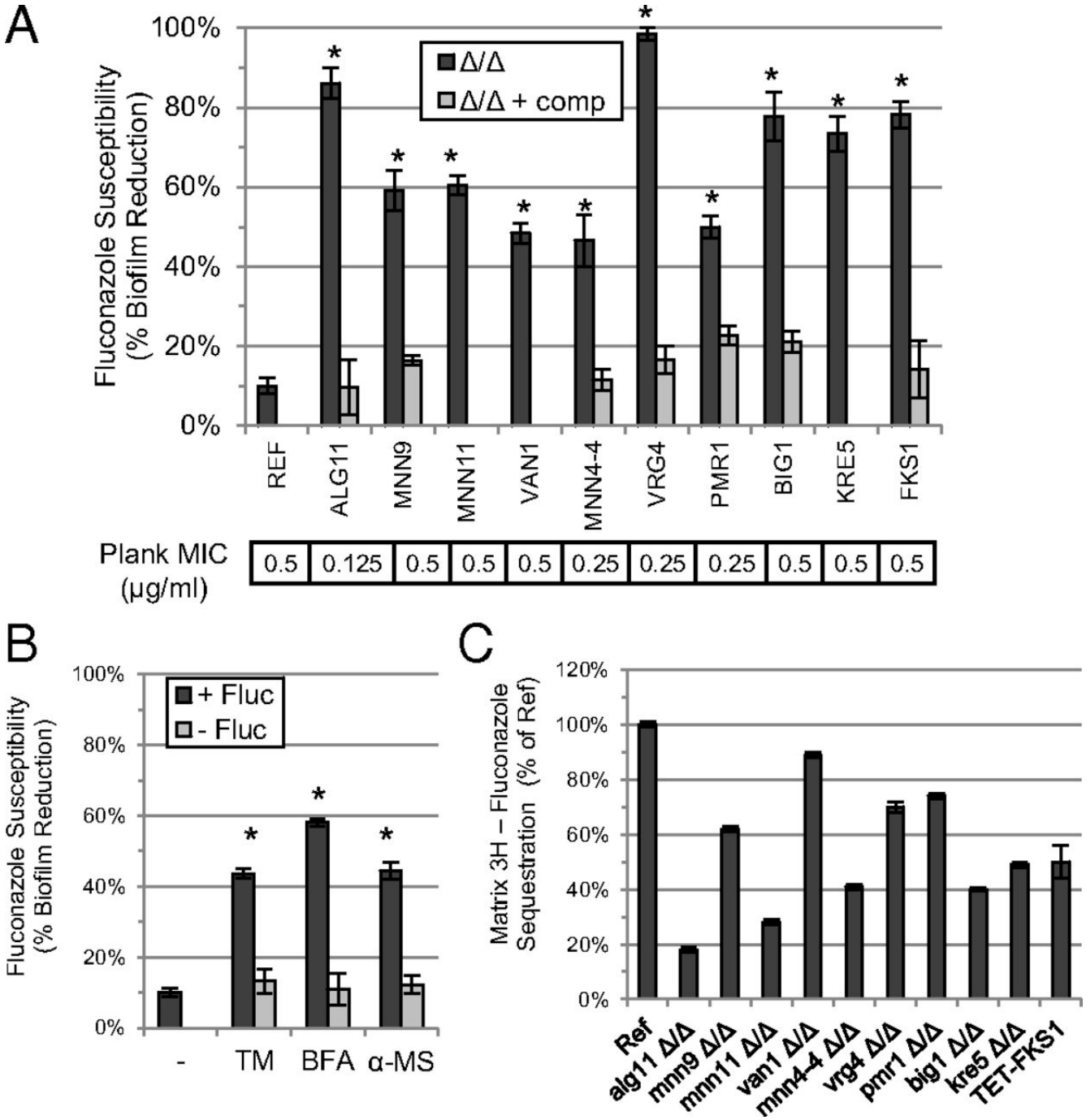

A
100%
80%
60%
40%
20%
0%
Fluconazole Susceptibility (% Biofilm Reduction)
Δ/Δ
Δ/Δ + comp
REF
ALG11
MNN9
MNN11
VAN1
MNN4-4
VRG4
PMR1
BIG1
KRE5
FKS1
Plank MIC (µg/ml)
0.5
0.125
0.5
0.5
0.5
0.25
0.25
0.25
0.5
0.5
0.5
B
Fluconazole Susceptibility (% Biofilm Reduction)
+ Fluc
- Fluc
-
TM
BFA
α-MS
C
Matrix 3H – Fluconazole Sequestration (% of Ref)
120%
100%
80%
60%
40%
20%
0%
Ref
alg11 Δ/Δ
mnn9 Δ/Δ
mnn11 Δ/Δ
van1 Δ/Δ
mnn4-4 Δ/Δ
vrg4 Δ/Δ
pmr1 Δ/Δ
big1 Δ/Δ
kre5 Δ/Δ
TET-FKS1

Figure 4

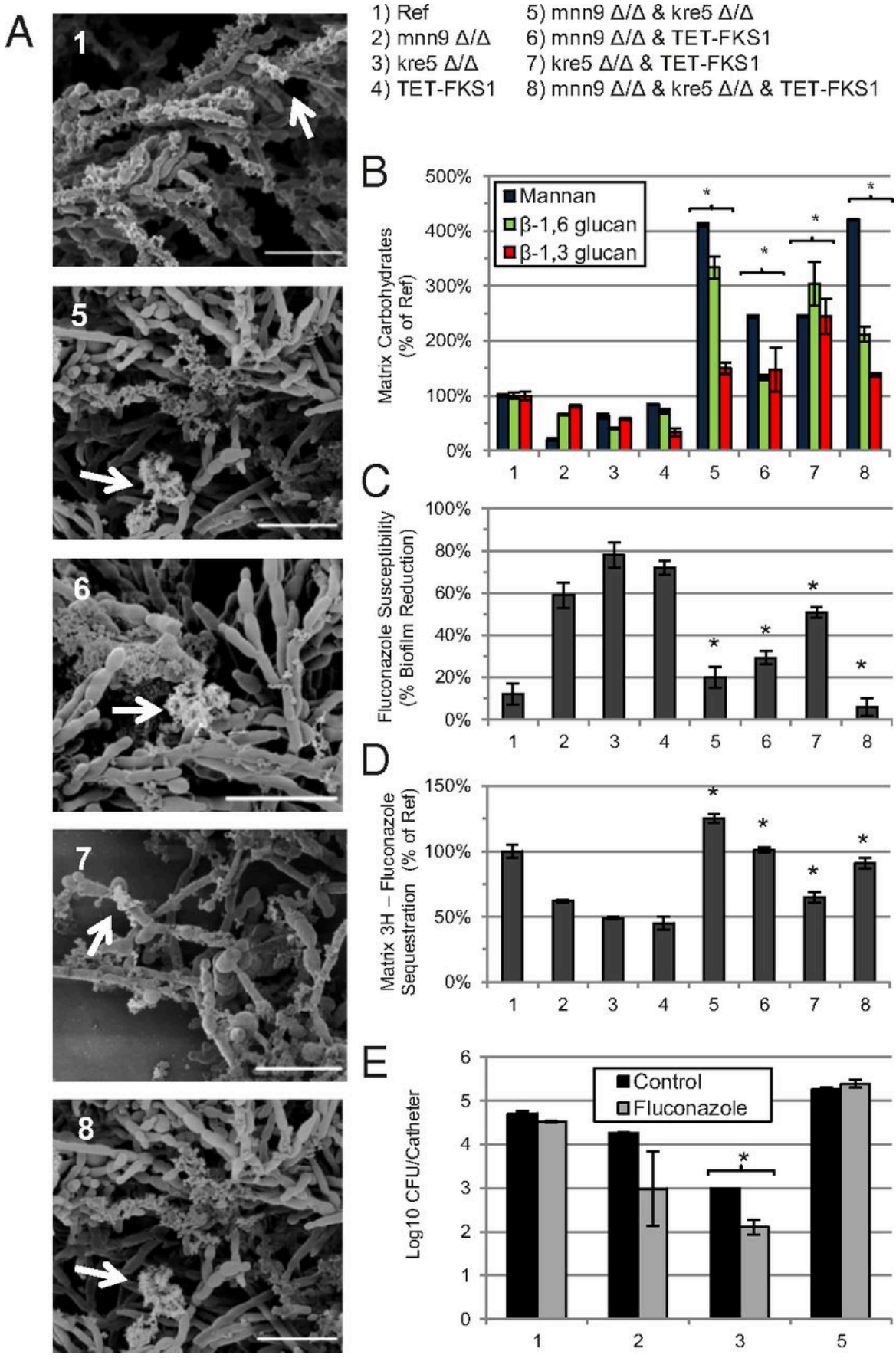

A
1
5
6
7
8
1) Ref
2) mnn9 Δ/Δ
3) kre5 Δ/Δ
4) TET-FKS1
5) mnn9 Δ/Δ & kre5 Δ/Δ
6) mnn9 Δ/Δ & TET-FKS1
7) kre5 Δ/Δ & TET-FKS1
8) mnn9 Δ/Δ & kre5 Δ/Δ & TET-FKS1
B
Matrix Carbohydrates (% of Ref)
Mannan
β-1,6 glucan
β-1,3 glucan
C
Fluconazole Susceptibility (% Biofilm Reduction)
D
Matrix 3H – Fluconazole Sequestration (% of Ref)
E
Log10 CFU/Catheter
Control
Fluconazole

Figure S1

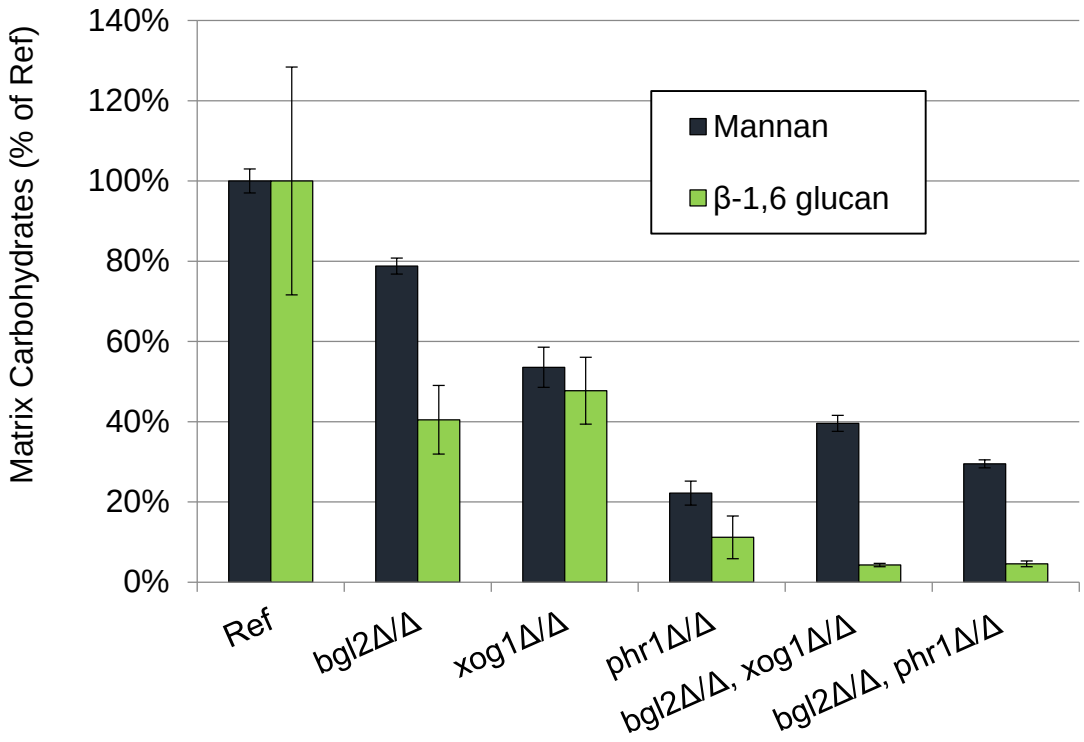
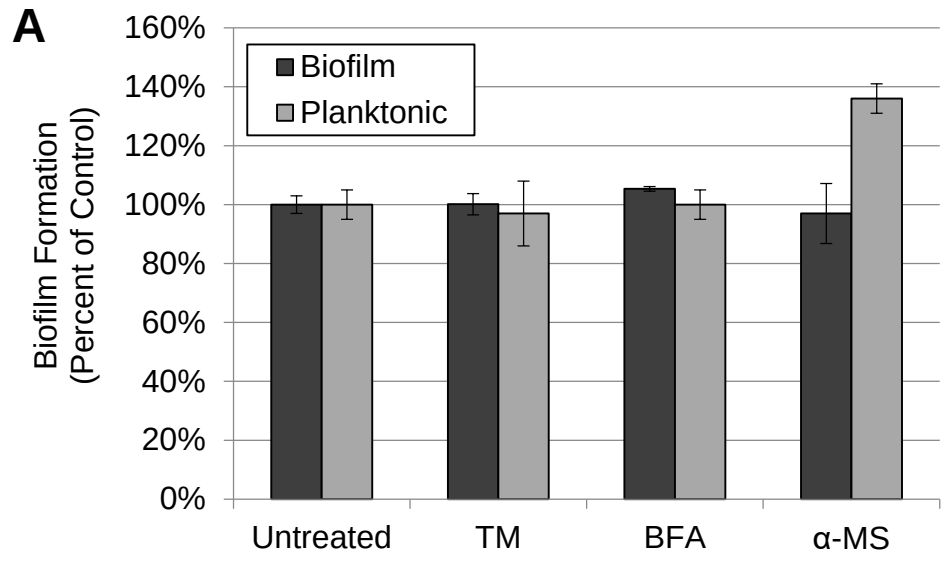

Matrix Carbohydrates (% of Ref)
140%
120%
100%
80%
60%
40%
20%
0%
Mannan
β-1,6 glucan
Ref
bgl2Δ/Δ
xog1Δ/Δ
phr1Δ/Δ
bgl2Δ/Δ, xog1Δ/Δ
bgl2Δ/Δ, phr1Δ/Δ

Figure S2


A
160%
140%
120%
100%
80%
60%
40%
20%
0%
Biofilm Formation (Percent of Control)
Biofilm
Planktonic
Untreated
TM
BFA
α-MS

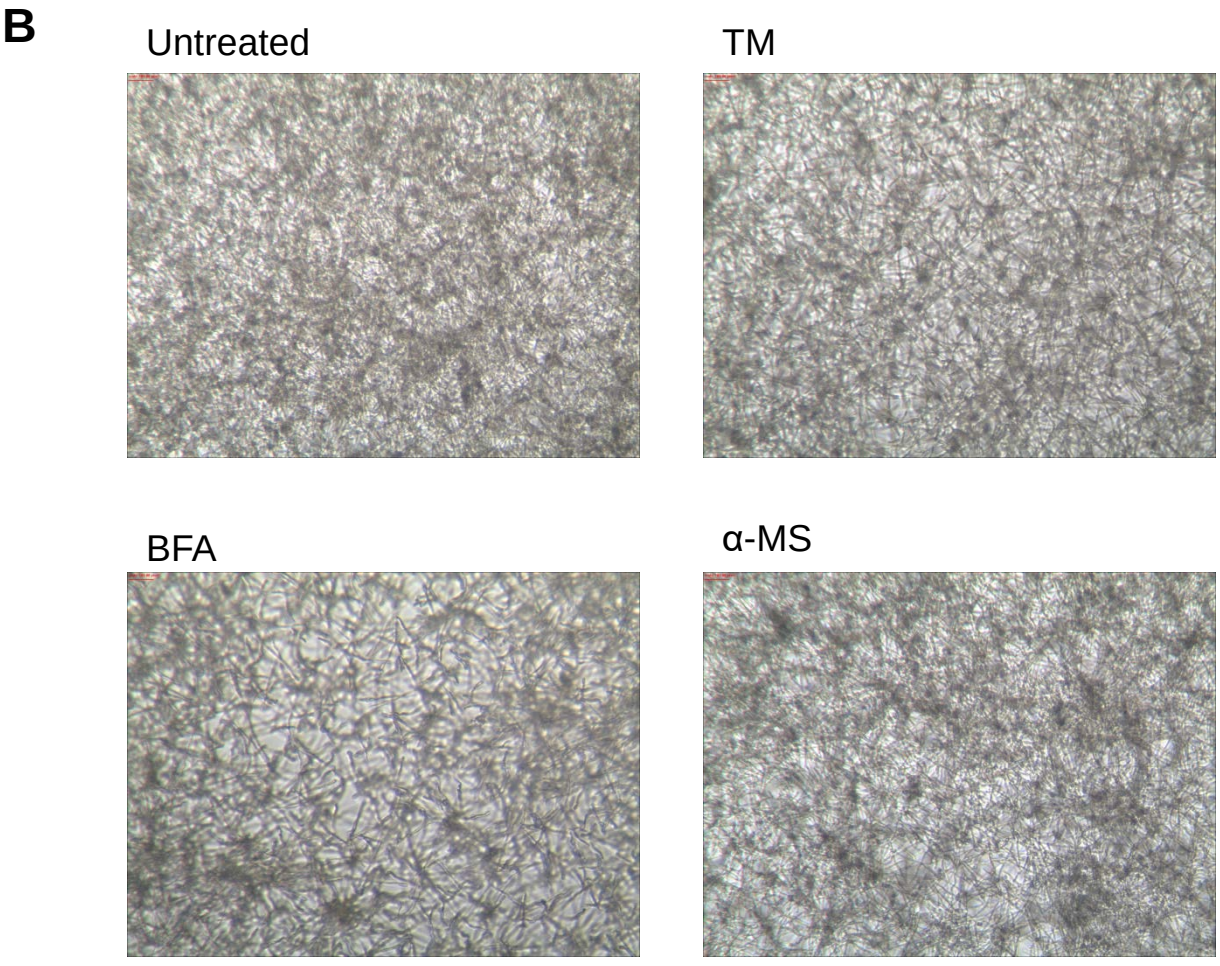
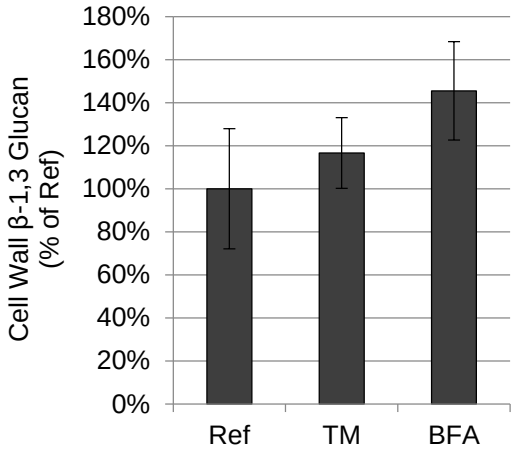

B
Untreated
TM
BFA
α-MS

Figure S3

A


Cell Wall β-1,3 Glucan (% of Ref)
180%
160%
140%
120%
100%
80%
60%
40%
20%
0%
Ref
TM
BFA

B

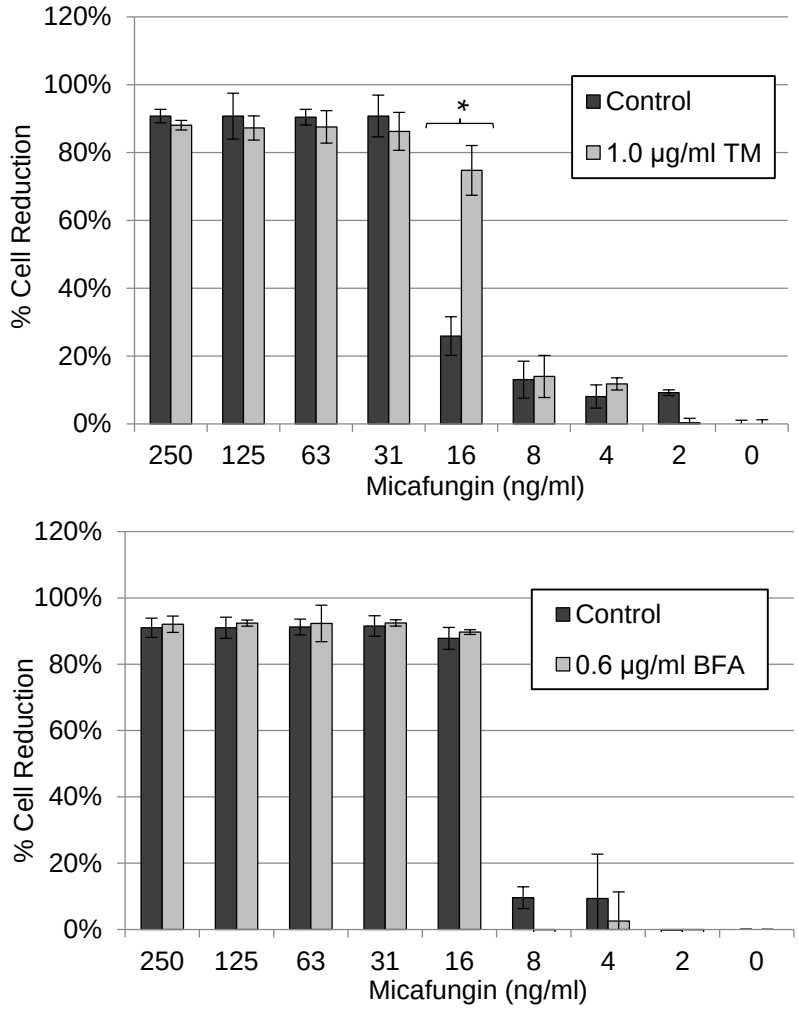

% Cell Reduction
120%
100%
80%
60%
40%
20%
0%
*
Control
1.0 µg/ml TM
250
125
63
31
16
8
4
2
0
Micafungin (ng/ml)
% Cell Reduction
Control
0.6 µg/ml BFA
Micafungin (ng/ml)

Figure S4

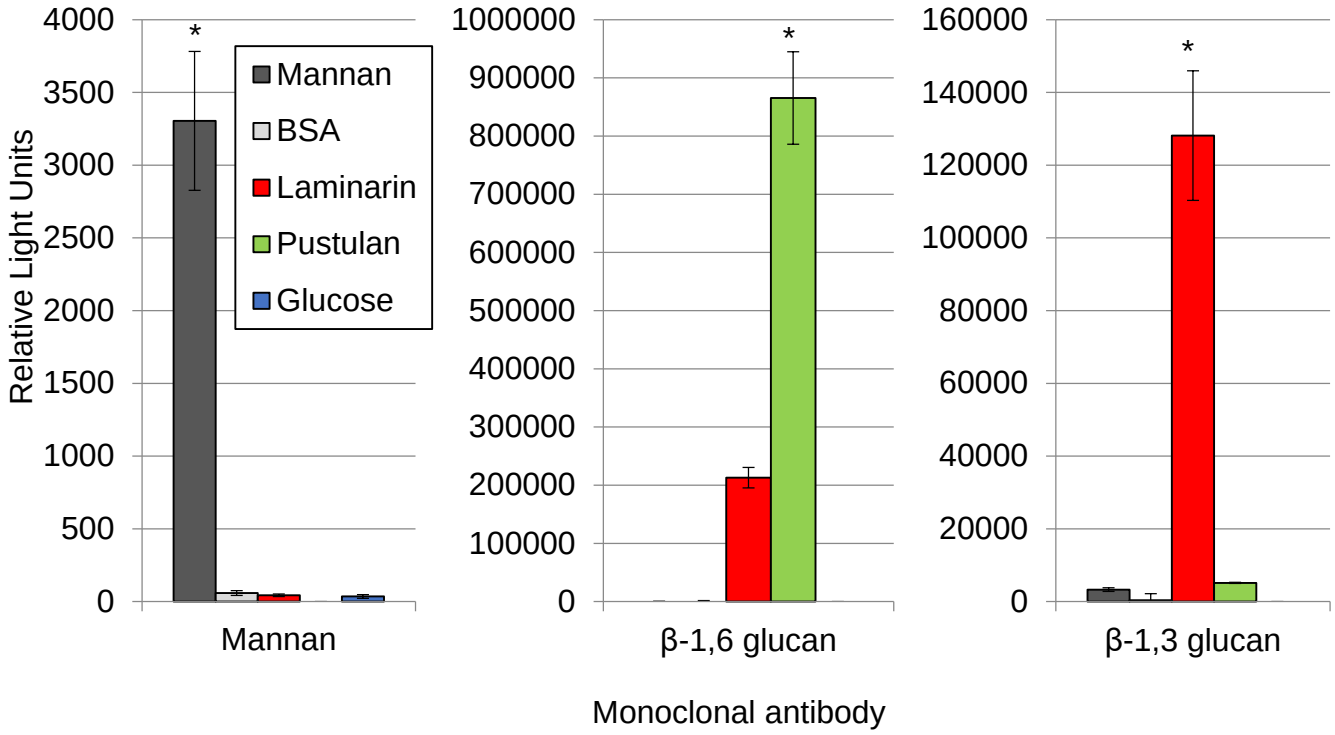

Relative Light Units
4000
3500
3000
2500
2000
1500
1000
500
0
Mannan
BSA
Laminarin
Pustulan
Glucose
*
1000000
900000
800000
700000
600000
500000
400000
300000
200000
100000
0
160000
140000
120000
100000
80000
60000
40000
20000
Mannan
β-1,6 glucan
β-1,3 glucan
Monoclonal antibody

Figure S5

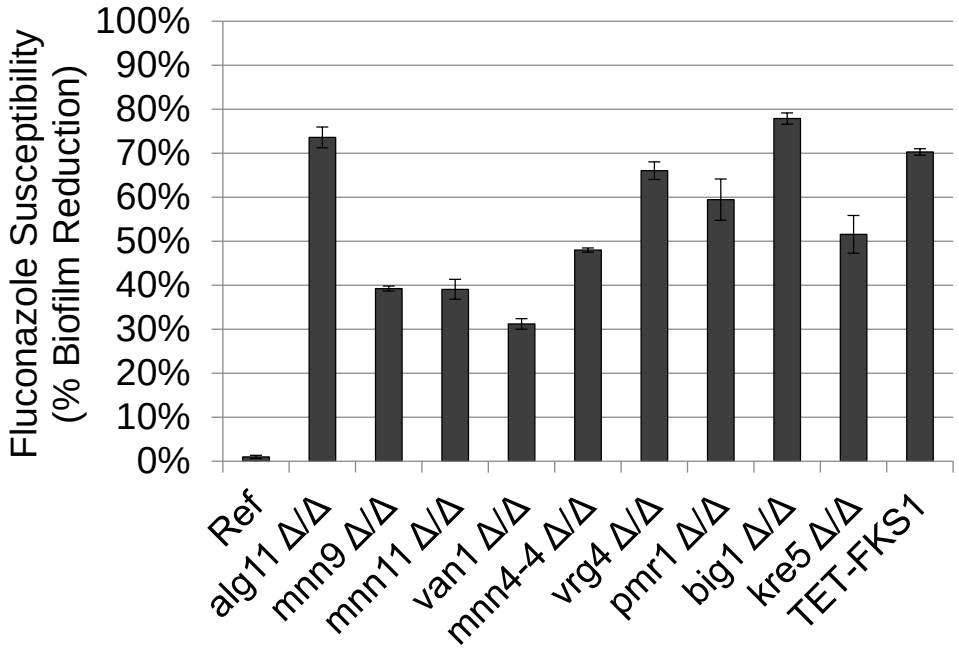


Figure S6

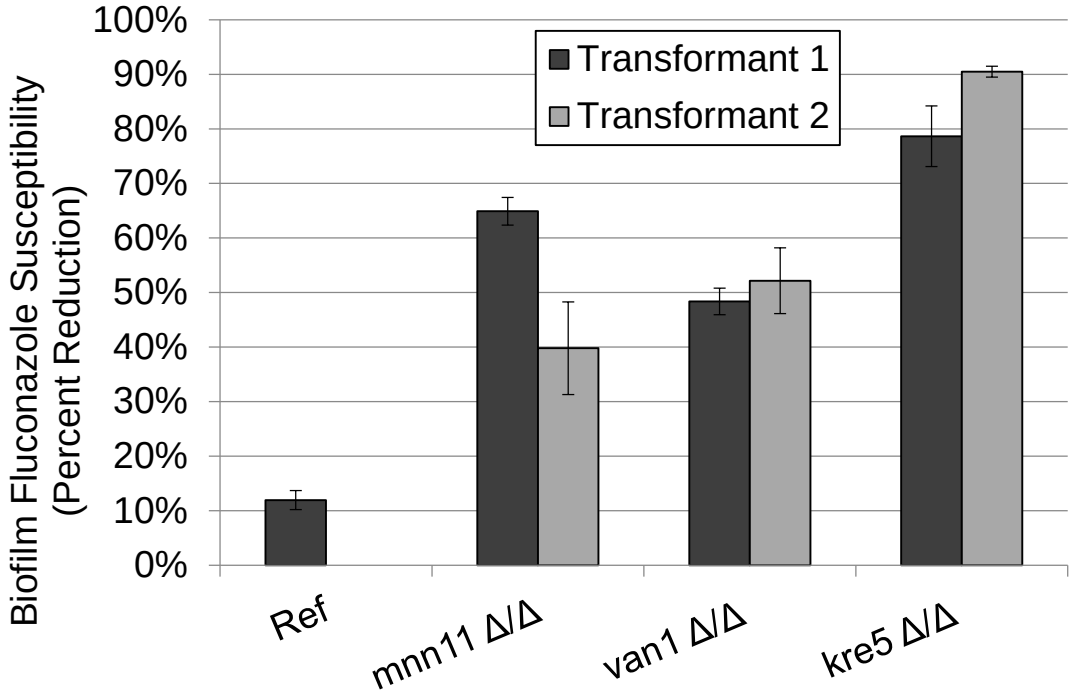


Figure S7

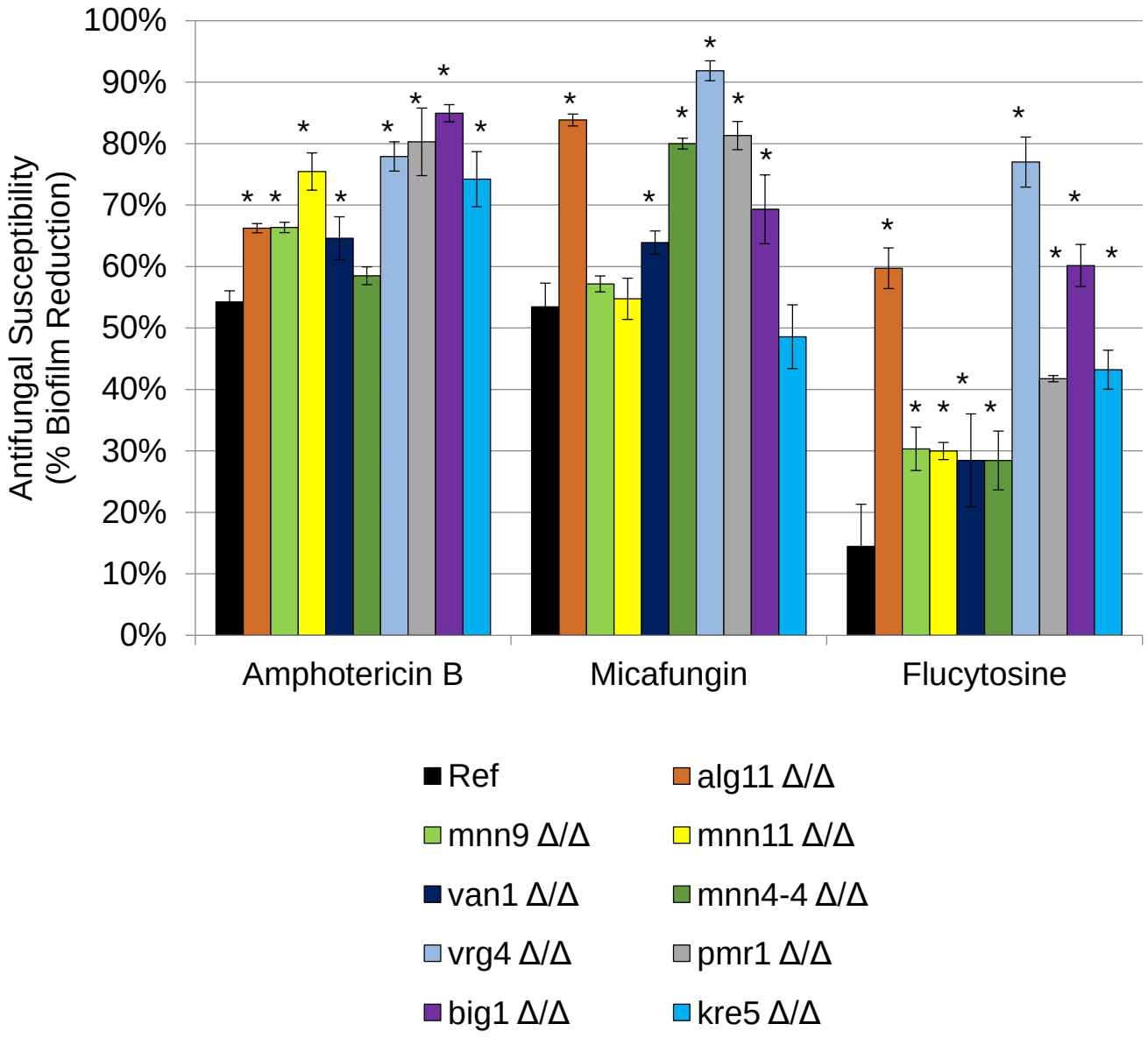

Antifungal Susceptibility
(% Biofilm Reduction)
100%
90%
80%
70%
60%
50%
40%
30%
20%
10%
0%
Amphotericin B
Micafungin
Flucytosine
Ref
alg11 Δ/Δ
mnn9 Δ/Δ
mnn11 Δ/Δ
van1 Δ/Δ
mnn4-4 Δ/Δ
vrg4 Δ/Δ
pmr1 Δ/Δ
big1 Δ/Δ
kre5 Δ/Δ

Figure S8

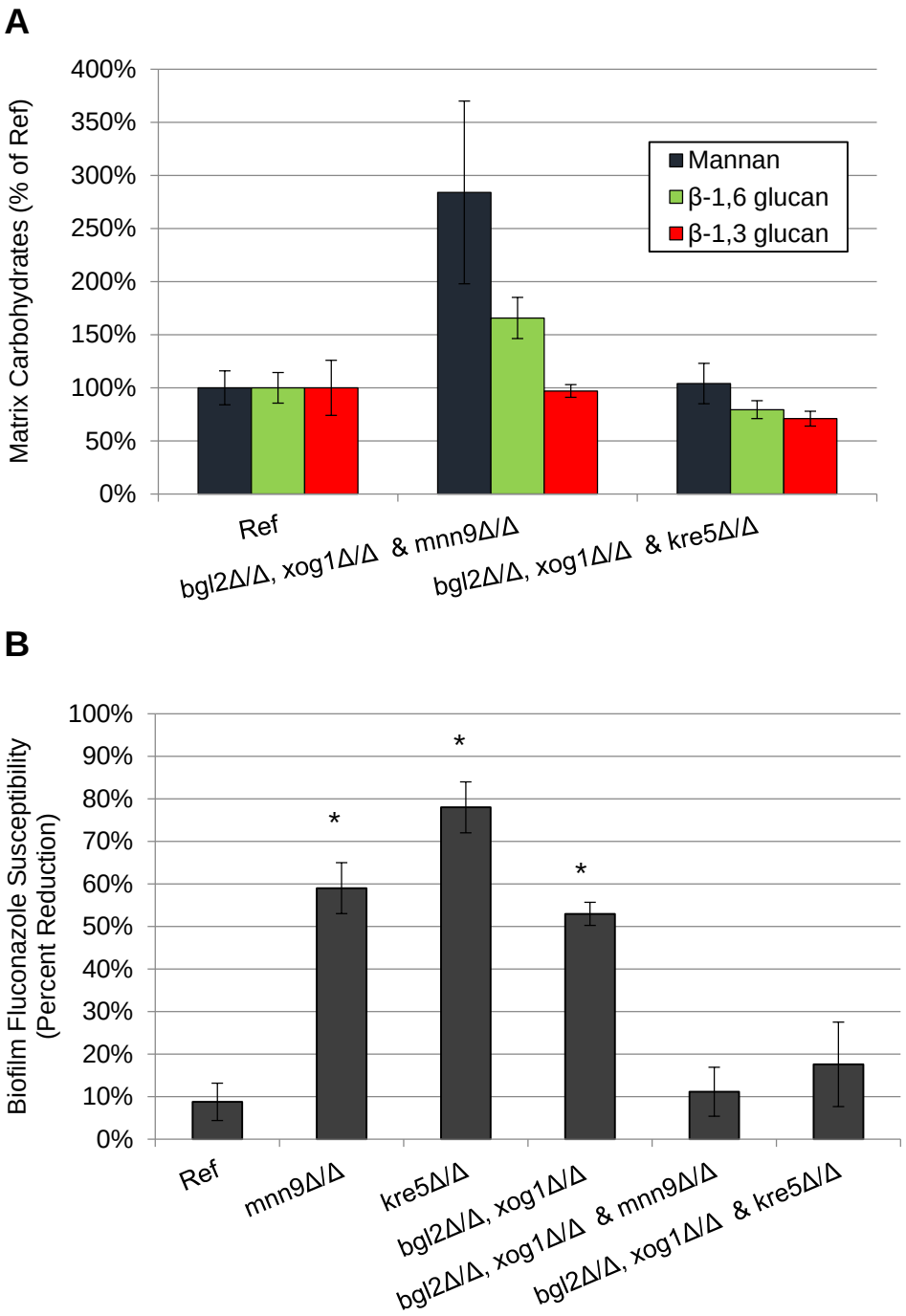
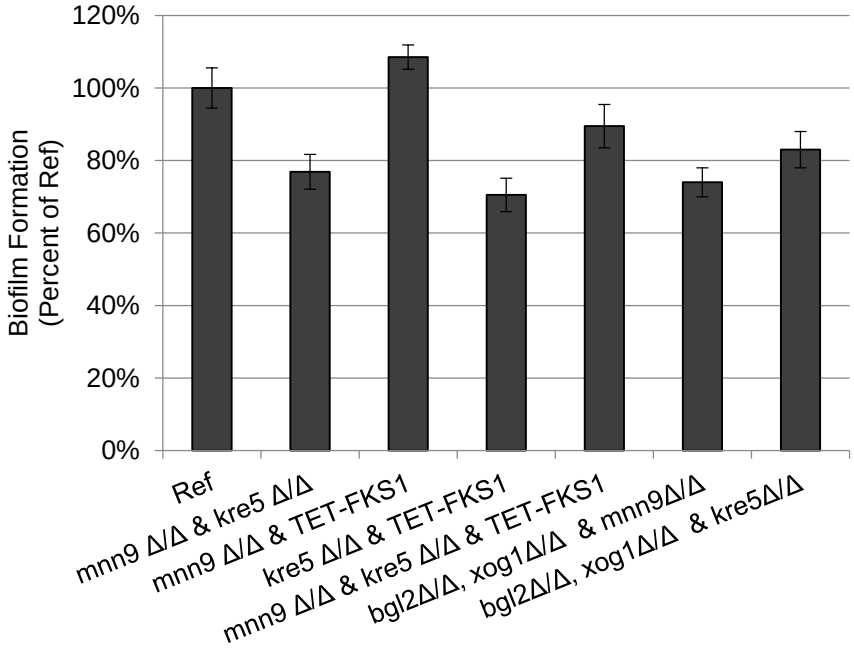

A
Matrix Carbohydrates (% of Ref)
400%
350%
300%
250%
200%
150%
100%
50%
0%
Mannan
β-1,6 glucan
β-1,3 glucan
Ref
bgl2Δ/Δ, xog1Δ/Δ & mnn9Δ/Δ
bgl2Δ/Δ, xog1Δ/Δ & kre5Δ/Δ
B
Biofilm Fluconazole Susceptibility (Percent Reduction)
100%
90%
80%
70%
60%
50%
40%
30%
20%
10%
0%
*
*
*
Ref
mnn9Δ/Δ
kre5Δ/Δ
bgl2Δ/Δ, xog1Δ/Δ
bgl2Δ/Δ, xog1Δ/Δ & mnn9Δ/Δ
bgl2Δ/Δ, xog1Δ/Δ & kre5Δ/Δ

Figure S9

A


120%
100%
80%
60%
40%
20%
0%
Biofilm Formation (Percent of Ref)
Ref
mnn9 Δ/Δ & kre5 Δ/Δ
mnn9 Δ/Δ & TET-FKS1
kre5 Δ/Δ & TET-FKS1
mnn9 Δ/Δ & kre5 Δ/Δ & TET-FKS1
bgl2Δ/Δ, xog1Δ/Δ & mnn9Δ/Δ
bgl2Δ/Δ, xog1Δ/Δ & kre5Δ/Δ

B

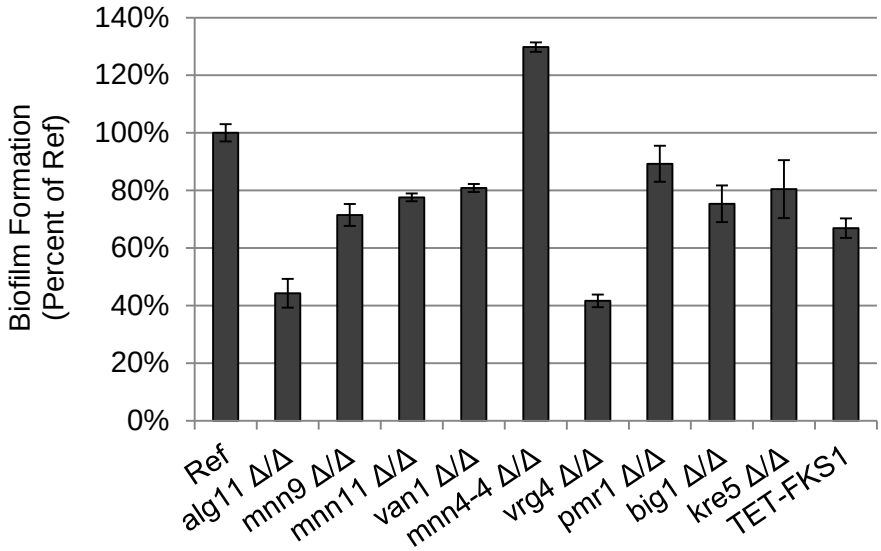

140%
120%
100%
80%
60%
40%
20%
0%
Biofilm Formation (Percent of Ref)
Ref
alg11 Δ/Δ
mnn9 Δ/Δ
mnn11 Δ/Δ
van1 Δ/Δ
mnn4-4 Δ/Δ
vrg4 Δ/Δ
pmr1 Δ/Δ
big1 Δ/Δ
kre5 Δ/Δ
TET-FKS1

Table S1. Mutant Strains Used in this Study

Gene Name	Systematic Name	Genotype	Strain Name	Description*
MNN2	19.2347	Δ/Δ	KMR391	α-1,2 mannosyltransferase
MNN21	19.1011	Δ/Δ	KFM148	α-1,2 mannosyltransferase
MNN22	19.3803	Δ/Δ	KFM132	α-1,2 mannosyltransferase
MNN23	19.4874	Δ/Δ	KMR350	α-1,2 mannosyltransferase
MNN24	19.1995	Δ/Δ	KFM113	α-1,2 mannosyltransferase
MNN26	19.6692	Δ/Δ	KMR383	α-1,2 mannosyltransferase
ALG11	19.3468	Δ/Δ	URZ217	α-1,2 mannosyltransferase
MNN9	19.7383	Δ/Δ	KMR392	α-1,6 mannosyltransferase
MNN10	19.5658	Δ/Δ	KMR338	α-1,6 mannosyltransferase
MNN11	19.2927	Δ/Δ	KMR347	α-1,6 mannosyltransferase
HOC1	19.3445	Δ/Δ	ELR117	α-1,6 mannosyltransferase
OCH1	19.7391	Δ/Δ	ELR125	α-1,6 mannosyltransferase
VAN1	19.6738	Δ/Δ	ELR108	α-1,6 mannosyltransferase
ANP1	19.3622	Δ/Δ	ELR107	α-1,6 mannosyltransferase
MNT4	19.4475	Δ/Δ	KMR336	mannosylphosphate transferase
MNN4	19.2881	Δ/Δ	KFM138	mannosylphosphate transferase
MNN4-4	19.5557	Δ/Δ	KMR384	mannosylphosphate transferase
MNN41	19.849	Δ/Δ	KMR351	mannosylphosphate transferase
MNN42	19.851	Δ/Δ	KMR349	mannosylphosphate transferase
MNN43	19.2957	Δ/+	KMR306	mannosylphosphate transferase
MNN44	19.2958	Δ/Δ	KMR380	mannosylphosphate transferase
MNN46	19.3110	Δ/Δ	KMR386	mannosylphosphate transferase
MNN47	19.4771	Δ/Δ	KMR341	mannosylphosphate transferase
MNT3	19.1010	Δ/Δ	KMR364	mannosylphosphate transferase
MNT5	19.4494	Δ/Δ	ELR118	mannosylphosphate transferase
DCW1	19.1989	Δ/Δ	ELR127	mannosidase
DFG5	19.2075	Δ/Δ	ELR129	mannosidase
AMS1	19.2768	Δ/Δ	ELR115	mannosidase
MNS1	19.1036	Δ/Δ	KFM107	mannosidase
VRG4	19.1232	Δ/Δ	URZ192	GDP-mannose transporter
PMR1	19.7089	Δ/Δ	KMR355	Ca^{2+}/Mn^{2+} ATPase
BMT1	19.6782	Δ/Δ	ELR138	β-mannosyltransferase
KRE1	19.4377	Δ/Δ	URZ266	β-1,6 glucan synthesis
KRE5	19.29	Δ/Δ	JAE104	β-1,6 glucosyltransferase
KRE6	19.7363	Δ/Δ	URZ270	β-1,6 glucan synthesis
KRE9	19.5861	Δ/Δ	JAE101	β-1,6 glucan synthesis
BIG1	19.2334	Δ/Δ	JAE103	β-1,6 glucan synthesis
SKN1	19.7362	Δ/Δ	URZ260	β-1,6 glucan synthesis
FKS1**	19.2929	Δ/+, repressible allele	TET-FKS1; MY2378A	β-1,3 glucan synthase
BGL2**	19.4565	Δ/Δ	HTT111	β-1,3 glucosyltransferase
XOG1**	19.2990	Δ/Δ	HTT117	β-1,3 glucanase
PHR1**	19.3829	Δ/Δ	KMR101	β-1,3 glucosyltransferase
BGL2,XOG1**		Δ/Δ, Δ/Δ	URZ290	double mutant
BGL2,PHR1**		Δ/Δ, Δ/Δ	URZ296	double mutant

*Based upon *Candida* or *Saccharomyces* Genome Database
**Mutant strains previously described in refs. 1 and 2

1. Roemer T, et al. (2003) Large scale essential gene e identification in Candida albicans and applications to antifungal drug discovery. Mol Microbiol 50(1):167–181.
2. Taff HT, et al. (2012) A Candida biofilm-induced pathway for matrix glucan delivery: Implications for drug resistance. PLoS Pathog 8(8):e1002848.

Table S2. Mutant Strains Developed in this Study

Gene	Strain	Genotype	Ref
Reference	SN152	*URA3* *IRO1* *arg4* *his1* *leu2* *ura3::λimm434* *iro1:: λimm434* *arg4* *his1* *leu2*	[41]
alg11 -/-	URZ216	*URA3* *IRO1* *arg4* *his1* *leu2* *alg11::C.d HIS1* *ura3::λimm434* *iro1:: λimm434* *arg4* *his1* *leu2* *alg11::C.m LEU2*	This study
alg11 -/-	URZ217	*URA3* *IRO1* *arg4* *his1* *leu2* *alg11::C.d HIS1* *ura3::λimm434* *iro1:: λimm434* *arg4* *his1* *leu2* *alg11::C.m LEU2*	This study
alg11 -/-	URZ218	*URA3* *IRO1* *arg4* *his1* *leu2* *alg11::C.d HIS1* *ura3::λimm434* *iro1:: λimm434* *arg4* *his1* *leu2* *alg11::C.m LEU2*	This study
alg11 -/-, +	KFM151	*URA3* *IRO1* *arg4* *his1* *leu2::ALG11::C.d ARG4* *alg11::C.d HIS1* *ura3::λimm434* *iro1:: λimm434* *arg4* *his1* *leu2* *alg11::C.m LEU2*	This study
mnn9 -/-	KMR392	*URA3* *IRO1* *arg4* *his1* *leu2* *mnn9::C.d HIS1* *ura3::λimm434* *iro1:: λimm434* *arg4* *his1* *leu2* *mnn9::C.m LEU2*	This study
mnn9 -/-	KMR393	*URA3* *IRO1* *arg4* *his1* *leu2* *mnn9::C.d HIS1* *ura3::λimm434* *iro1:: λimm434* *arg4* *his1* *leu2* *mnn9::C.m LEU2*	This study
mnn9 -/-, +	KFM137	*URA3* *IRO1* *arg4* *his1* *leu2::MNN9::C.d ARG4* *mnn9::C.d HIS1* *ura3::λimm434* *iro1:: λimm434* *arg4* *his1* *leu2* *mnn9::C.m LEU2*	This study
mnnn11 -/-	KMR346	*URA3* *IRO1* *arg4* *his1* *leu2* *mnn11::C.d HIS1* *ura3::λimm434* *iro1:: λimm434* *arg4* *his1* *leu2* *mnn11::C.m LEU2*	This study
mnn11 -/-	KMR347	*URA3* *IRO1* *arg4* *his1* *leu2* *mnn11::C.d HIS1* *ura3::λimm434* *iro1:: λimm434* *arg4* *his1* *leu2* *mnn11::C.m LEU2*	This study
van1 -/-	ELR108	*URA3* *IRO1* *arg4* *his1* *leu2* *van1::C.d HIS1* *ura3::λimm434* *iro1:: λimm434* *arg4* *his1* *leu2* *van1::C.m LEU2*	This study
van1 -/-	ELR111	*URA3* *IRO1* *arg4* *his1* *leu2* *van1::C.d HIS1* *ura3::λimm434* *iro1:: λimm434* *arg4* *his1* *leu2* *van1::C.m LEU2*	This study
mnn4-4 -/-	KMR384	*URA3* *IRO1* *arg4* *his1* *leu2* *mnn4-4::C.d HIS1* *ura3::λimm434* *iro1:: λimm434* *arg4* *his1* *leu2* *mnn4-4::C.m LEU2*	This study
mnn4-4 -/-	KMR385	*URA3* *IRO1* *arg4* *his1* *leu2* *mnn4-4::C.d HIS1* *ura3::λimm434* *iro1:: λimm434* *arg4* *his1* *leu2* *mnn4-4::C.m LEU2*	This study
mnn4-4 -/-, +	KFM166	*URA3* *IRO1* *arg4* *his1* *leu2::MNN4-4::C.d ARG4* *mnn4-4::C.d HIS1* *ura3::λimm434* *iro1:: λimm434* *arg4* *his1* *leu2* *mnn4-4::C.m LEU2*	This study

vrg4 -/-	URZ192	*URA3 IRO1 arg4 his1 leu2 vrg4::C.d HIS1* *ura3::λimm434 iro1:: λimm434 arg4 his1 leu2 vrg4::C.m LEU2*	This study
vrg4 -/-	URZ193	*URA3 IRO1 arg4 his1 leu2 vrg4::C.d HIS1* *ura3::λimm434 iro1:: λimm434 arg4 his1 leu2 vrg4::C.m LEU2*	This study
vrg4 -/-, +	KFM158	*URA3 IRO1 arg4 his1 leu2::VRG4::C.d ARG4 vrg4::C.d HIS1* *ura3::λimm434 iro1:: λimm434 arg4 his1 leu2 vrg4::C.m LEU2*	This study
pmr1 -/-	KMR354	*URA3 IRO1 arg4 his1 leu2 pmr1::C.d HIS1* *ura3::λimm434 iro1:: λimm434 arg4 his1 leu2 pmr1::C.m LEU2*	This study
pmr1 -/-	KMR355	*URA3 IRO1 arg4 his1 leu2 pmr1::C.d HIS1* *ura3::λimm434 iro1:: λimm434 arg4 his1 leu2 pmr1::C.m LEU2*	This study
pmr1 -/-, +	KFM150	*URA3 IRO1 arg4 his1 leu2::PMR1::C.d ARG4 pmr1::C.d HIS1* *ura3::λimm434 iro1:: λimm434 arg4 his1 leu2 pmr1::C.m LEU2*	This study
big1 -/-	JAE103	*URA3 IRO1 arg4 his1 leu2 big1::C.d HIS1* *ura3::λimm434 iro1:: λimm434 arg4 his1 leu2 big1::C.m LEU2*	This study
big1 -/-, +	JAE119	*URA3 IRO1 arg4 his1 leu2::BIG1::C.d ARG4 big1::C.d HIS1* *ura3::λimm434 iro1:: λimm434 arg4 his1 leu2 big1::C.m LEU2*	This study
kre5 -/-	JAE104	*URA3 IRO1 arg4 his1 leu2 kre5::C.d HIS1* *ura3::λimm434 iro1:: λimm434 arg4 his1 leu2 kre5::C.m LEU2*	This study
kre5 -/-	JAE109	*URA3 IRO1 arg4 his1 leu2 kre5::C.d HIS1* *ura3::λimm434 iro1:: λimm434 arg4 his1 leu2 kre5::C.m LEU2*	This study

Table S3. Primers for Mutant Strain Creation

Gene	Function	Primers
ALG11	Knockout	Upstream F: 5' – TCCTCCTCCTCCTTTTTACTTTTT Upstream R: 5' – GTCAGCGGCCGCATCCCTGCTTTGCATTTGGTCTTAACTTCTTG Auxotrophic marker F: 5' – CCGCTGCTAGGCGCGCCGTGACCAGTGTGATGGATATCTGC Auxotrophic marker R: 5' – GCAGGGATGCGGCCGCTGACAGCTCGGATCCACTAGTAACG Downstream F: 5' – GTCAGCGGCCGCATCCCTGCTTTGCATTTGGTCTTAACTTCTTG Downstream R: 5' – TTGATGACCTTCAAAAGTTTGTTC Nested Fusion F: 5' – AAGAAGAAGAAAAGGGGAAGTTGT Nested Fusion R: 5' – TTTTTACGAGCAATGAACTTACCA Upstream Check F: 5' – ATGTGTTTTATATGCAGTTCCA Downstream Check R: 5' – AAATGTACGGTTTAGAAGGATG Internal Check F: 5' – TGCCTATACTCATTTCCCCATATT Internal Check R: 5' – TCTGTTTTGATCGATTTGTCTCAT
ALG11	Complement	BamHI F: 5' – AGGATCCTTCCTCCTCCTCCTTTTTAC AscI R: 5' – AGGCGCGCC CTATCTAAACATGCCGAAGC
MNN9	Knockout	Upstream F: 5' – CAACATGTTTGCTTGGGATG Upstream R: 5' – CACGGCGCGCCTAGCAGCGGTGGAGCGTTGAAAGTAAGACAA Auxotrophic marker F: 5' – CCGCTGCTAGGCGCGCCGTGACCAGTGTGATGGATATCTGC Auxotrophic marker R: 5' – GCAGGGATGCGGCCGCTGACAGCTCGGATCCACTAGTAACG Downstream F: 5' – GTCAGCGGCCGCATCCCTGCCTCCCATTGAGTTTGGGTGT Downstream R: 5' – CTACCCAAAGCAGCACGTTT Nested Fusion F: 5' – ATAATAAATTGATTGTTTTGTTGTTGTTCT Nested Fusion R: 5' – TTAACTTTTCTCATATAAAAAGCACGAATA Upstream Check F: 5' – CAGATTGTCTGCTTCCACCA Downstream Check R: 5' – TTGGCAATCTACCCAAATGTT Internal Check F: 5' – CTTTTCTGCCCATGGGTTT Internal Check R: 5' – TAACTGCACCTCCACCAACA
MNN9	Complement	AscI F: 5' – AGGCGCGCCAATGATGAATTGACTTTAAC BamHI R: 5' – AGGATCCCTACCCAAAGCAGCACGTTT
MNN11	Knockout	Upstream F: 5' – AGAAACTGCTGCAAATGGTG Upstream R: 5' – CACGGCGCGCCTAGCAGCGGCATCGTTCGGTTCGTTCATT Auxotrophic marker F: 5' – CCGCTGCTAGGCGCGCCGTGACCAGTGTGATGGATATCTGC Auxotrophic marker R: 5' – GCAGGGATGCGGCCGCTGACAGCTCGGATCCACTAGTAACG Downstream F: 5' – GTCAGCGGCCGCATCCCTGC GGGACCCCCACCTTACTACT Downstream R: 5' – CAACAACACCAAATAGCACCA Nested Fusion F: 5' – AGTAGTAGTGAATAACCAATCATGAGAATG Nested Fusion R: 5' – AATCATACTTAAAAGTGATAGCAATGGTAG Upstream Check F: 5' – CGAGCACAAAAGAGACAAGAA Downstream Check R: 5' – GTGGTGGTGGTGGAACTGAT Internal Check F: 5' – CAATTCATTTTTATCACCATTTTCA Internal Check R: 5' – TGACTAGCAGCAATAGTTTTAATTGG
VAN1	Knockout	Upstream F: 5' – GTCGATCACACTCCCGTTTT Upstream R: 5' – CACGGCGCGCCTAGCAGCGGTTGTATTCCCTTTTAGAGGTAG Auxotrophic marker F: 5' – CCGCTGCTAGGCGCGCCGTGACCAGTGTGATGGATATCTGC Auxotrophic marker R: 5' – GCAGGGATGCGGCCGCTGACAGCTCGGATCCACTAGTAACG Downstream F: 5' – GTCAGCGGCCGCATCCCTGCCAATTCTGATCGTAAAAAT Downstream R: 5' – CAGTGTTTTGAGAGGCAATCC Nested Fusion F: 5' – AGATTTACTTGCAGAGGGTCT Nested Fusion R: 5' – TACGTACCAAGAAGCATCTAATC Upstream Check F: 5' – TGGTATTTCGGCTGCTACAA Downstream Check R: 5' – ATGGGTTTGTAAATGGTCTACTTTT Internal Check F: 5' – TCACCGACCAACGACAATAA Internal Check R: 5' – CGCCAATTCTAATCCAGCAT
MNN4-4	Knockout	Upstream F: 5' – AAATGCAATCAAACCGCTCT Upstream R: 5' – CACGGCGCGCCTAGCAGCGGTACACCCACACACACGCAAA Auxotrophic marker F: 5' – CCGCTGCTAGGCGCGCCGTGACCAGTGTGATGGATATCTGC Auxotrophic marker R: 5' – GCAGGGATGCGGCCGCTGACAGCTCGGATCCACTAGTAACG Downstream F: 5' – GTCAGCGGCCGCATCCCTGC CCCACTTCTTTTCCCATACG Downstream R: 5' – AACAACACAATGCCAACAGC Nested Fusion F: 5' – AAAGCATTAGCGGTGGTAGTAGTAGTAGTC Nested Fusion R: 5' – CTATTCTGAATTGTTAGGTCAATTAGGTC Upstream Check F: 5' – CCCATTGGTCAGAGTTTTCC Downstream Check R: 5' – TTTCCGAAATGTGGGTTTTG Internal Check F: 5' – GTGTAATGCAAGCCAAAGCA Internal Check R: 5' – TTCCCGTATCCTTCACTTGG
MNN4-4	Complement	AscI F: 5' – AGGCGCGCCTTTTCCGTGTTTAGTTAATA

		BamHI R: 5’ – AGGATCCTTTCCGAAATGTGGGTTTTG
VRG4	Knockout	Upstream F: 5’ – TGAAATAACTTGGTAGACTTCAGATTG Upstream R: 5’ – CACGGCGCGCCTAGCAGCGGACAATGGTTTAGTAGGAAGGGAAG Auxotrophic marker F: 5’ – CCGCTGCTAGGCGCGCCGTGACCAGTGTGATGGATATCTGC Auxotrophic marker R: 5’ – GCAGGGATGCGGCCGCTGACAGCTCGGATCCACTAGTAACG Downstream F: 5’ – GTCAGCGGCCGCATCCCTGCTTTATTATCAATTGAGGAAGGAAAAA Downstream R: 5’ – AAACTTCTTCTTGAGTGCCCTTTA Nested Fusion F: 5’ – TTTGATTTGGTAGATATTACACACGA Nested Fusion R: 5’ – GACAATACAACCTCCCCATTGATA Upstream Check F: 5’ – GGCTAGACTGTTGGTGTTGT Downstream Check R: 5’ – GCCTAGTTTGTTTTGTTTGG Internal Check F: 5’ – TTCTTCTTCTTCTTCTGGCTCATT Internal Check R: 5’ – CAACAGCATAAACTAATCCTGCAC
VRG4	Complement	BamHI F: 5’ – AGGATCCCAATTCTACTTTCTTGGTGTATTG AscI R: 5’ – AGGCGCGCCTTCTTCTTGAGTGCCCTTTA
PMR1	Knockout	Upstream F: 5’ – TTCACCAACAGCAAAAGTGG Upstream R: 5’ – CACGGCGCGCCTAGCAGCGGAGGTCTGCGTGGTGTTTTCT Auxotrophic marker F: 5’ – CCGCTGCTAGGCGCGCCGTGACCAGTGTGATGGATATCTGC Auxotrophic marker R: 5’ – GCAGGGATGCGGCCGCTGACAGCTCGGATCCACTAGTAACG Downstream F: 5’ – GTCAGCGGCCGCATCCCTGC CGAGTGGAGAGGGGATTGTA Downstream R: 5’ – GCTAAGGGTACTTGCGATGC Nested Fusion F: 5’ – ATTGAAAATTATTTGACCAACAATAAAGTA Nested Fusion R: 5’ – CATTATCTATAGTGAATTTGAAAACCATTG Upstream Check F: 5’ – CGACCAGGAGGTGTTTCATT Downstream Check R: 5’ – CCTGTCCGGTTTTGCTTAAA Internal Check F: 5’ – GAATCCCCGCAGACATTAGA Internal Check R: 5’ – ATGCACACCACCTTGCATTA
PMR1	Complement	AscI F: 5’ – AGGCGCGCCCGACCAGGAGGTGTTTCATT AscI R: 5’ – AGGCGCGCCCCTGTCCGGTTTTGCTTAAA Upstream Orientation Check F: 5’ – GAAAAAGATGTTATGGAAACT Upstream Orientation Check R: 5’ – ATGACCGCTAGTCCTTCGTTT Downstream Orientation Check F: 5’ – GGCTCAAAAAGTTATTGCCGA Downstream Orientation Check R: 5’ – CCGCCACCACCACGACCACT
BIG1	Knockout	Upstream F: 5’ – AGGTTCAACATTAAGCTCTTTTGC Upstream R: 5’ – CACGGCGCGCCTAGCAGCGGATCTCAAGAAACGAACTTTTGAGC Auxotrophic marker F: 5’ – CCGCTGCTAGGCGCGCCGTGACCAGTGTGATGGATATCTGC Auxotrophic marker R: 5’ – GCAGGGATGCGGCCGCTGACAGCTCGGATCCACTAGTAACG Downstream F: 5’ – GTCAGCGGCCGCATCCCTGCTATTTCTTGCCCAATTCCTATTTC Downstream R: 5’ – ACTGCACAAGTTCATCCTTACAAA Nested Fusion F: 5’ – GCTGAGCTTTCCTTTTCATTTTTA Nested Fusion R: 5’ – TAGTCCGTATGCCATCTTTACTGA Upstream Check F: 5’ – CAATCAATTCCACCAAAGAT Downstream Check R: 5’ – GAAAGAGGACAAGCATCAAG Internal Check F: 5’ – TGTCAGTTAACTCCAGTGTTGGTT Internal Check R: 5’ – GCTTCGATTTTTGTTGTTTCTTTT
BIG1	Complement	AscI F: 5’ – AGGCGCGCCTGGTTCTTTCGTGAATTTCT BamHI R: 5’ – AGGATCCAACTGCACAAGTTCATCCTT
KRE5	Knockout	Upstream F: 5’ – CACCAATAATACACGAGCAGAAGT Upstream R: 5’ – CACGGCGCGCCTAGCAGCGGAAAACTAATAACAACGCATCCACA Auxotrophic marker F: 5’ – CCGCTGCTAGGCGCGCCGTGACCAGTGTGATGGATATCTGC Auxotrophic marker R: 5’ – GCAGGGATGCGGCCGCTGACAGCTCGGATCCACTAGTAACG Downstream F: 5’ – GTCAGCGGCCGCATCCCTGCTACGCACGTTGGGTAGAGATAATA Downstream R: 5’ – TACCCTGCAACACCAATATTAAAG Nested Fusion F: 5’ – CTAAATGTCAATCACGGACAAAAG Nested Fusion R: 5’ – TGGGCAGTATATACCATTTCATTG Upstream Check F: 5’ – TGATGCAGCAAGATTAGTTG Downstream Check R: 5’ – CTTTCAAATAGCGCAAAGTT Internal Check F: 5’ – AAAGTATTCCATCCAATGTGACCT Internal Check R: 5’ – TTCTTTCCAAAATCTGAAACCTTC

Chapter 3: Role of matrix polysaccharides in antifungal resistance of non-*albicans Candida* biofilms

Section 1: β-1,3 glucan

The contents of this section were published:

Kaitlin F. Mitchell, Heather T. Taff, Miguel A. Cuevas, Emily L. Reinicke, Hiram Sanchez, David R. Andes. Role of Matrix β-1,3 Glucan in Antifungal Resistance of Non-albicans *Candida* Biofilms. Antimicrob Agents Chemother. 2013 Apr;57(4):1918-20. PMCID: PMC3623361.

H. Taff assisted in drug susceptibility and ELISA testing. M. Cuevas performed initial drug susceptibility testing, with results confirmed by E. Reinicke. H. Sanchez consulted for some ELISA testing and for the drug sequestration experiment. I confirmed all ELISA and drug susceptibility assays and completed the drug sequestration experiment.

Abstract

Candida biofilm infections pose an increasing threat in the health care setting due to the drug resistance associated with this lifestyle. Several mechanisms underlie the resistance phenomenon. In *C. albicans,* one mechanism for this resistance involves drug impedance by the biofilm matrix linked to β-1,3 glucan in the matrix. Here we show this is important for other *Candida* spp. We identified β-1,3 glucan in the matrix, found the matrix sequesters antifungal drug, and enhanced antifungal susceptibility with matrix β-1,3 glucan hydrolysis.

Article

Similar to many microbes, *Candida* species exhibit a propensity to grow as biofilms on implanted medical devices such as a central venous catheter (1, 2). Among biofilm-forming pathogens, infection due to *Candida* spp is associated with the highest nosocomial mortality (3). Treating these proves challenging due to high levels of drug resistance (4, 5). Compared to their planktonic counterparts, biofilm cells exhibit up to a 1,000-fold increase in resistance (6, 7). For most patients, removal of the medical device is the only viable treatment option (1).

A number of factors contribute to *Candida albicans* biofilm resistance (8, 9, 10, 11). The extracellular matrix that enmeshes the biofilm cells accounts for a large percentage of this phenotype by sequestering antifungal drugs. The matrix polysaccharide β-1,3 glucan has been strongly linked to this mechanism (12, 13, 14).

While *C. albicans* remains the most frequently isolated *Candida* species, other members of the genus are increasingly common. The most recent surveillance data in the US (15) found *C. albicans* comprised far less than 50% of isolates. *C. glabrata* (29%), *C. parapsilosis* (17%), and *C. tropicalis* (10%) as a group represented the majority of infections. Each of these species has been shown to form biofilms with comparable levels of antifungal resistance to *Candida albicans* (16, 17, 18).

The increasing prevalence of non-*albicans Candida* species and their role in biofilm device infections prompted us to ask if they also exhibit a β-1,3 glucan matrix resistance mechanism. The purpose of this study was to determine if β-1,3 glucan was present in the matrix of these species and, if so, did it play a role in drug resistance similar to that described for *C. albicans.* Specifically, three experiments with three non-*albicans Candida* species were undertaken: (i) determination of matrix β-1,3 glucan content, (ii) assessment of the extracellular matrix to sequester the antifungal fluconazole, and (iii) examination of the impact of β-1,3 glucan disruption on biofilm antifungal drug susceptibility. `

C. glabrata, C. parapsilosis, and *C. tropicalis* were chosen for study based upon relative incidence in clinical surveillance and demonstrated propensity for device biofilm formation. With the exception of the *C. albicans* isolate (strain SN250), all strains were clinical isolates from cases of invasive candidiasis [*C. glabrata* (strains 570 and 5376), *C. parapsilosis* (strains 5986 and CD371), and *C. tropicalis* (strains 2058 and 98-234)]. Biofilms were grown in RPMI-MOPS media on a polystyrene substrate for all experiments. Each of the strains formed robust biofilms with an average XTT OD of 1.42 for *C. albicans* and 1.40 for the non-*albicans* group after a 24h incubation. For matrix composition analysis, biofilms were grown for 48h using 1L roller bottles. Matrix was isolated using water bath sonication and vortexing as previously described (19). A β-1,3 glucan ELISA was performed on three biologic replicates and assays were completed in triplicate for each strain as previously detailed. Matrix β-1,3 glucan was normalized by matrix dry weight and expressed as ng/mg matrix. As shown in Fig. 1A, the β-1,3 glucan polymer was identified in the biofilm matrix of each *Candida* strain tested. The concentration of this polysaccharide was relatively similar among the species.

We utilized a 6-well plate format for assessment of antifungal drug biofilm penetration using [H^3] fluconazole as described (13, 19). Briefly, mature biofilms (24 h incubation) were washed twice with sterile water followed by exposure to a total of $8.48x10^5$ cpm of [H^3] fluconazole in RPMI-MOPS media. Biofilms were then incubated for 30 min at 37^{o}C, and then chased with 20

μM unlabeled fluconazole in media. The fluconazole content was measured in intact biofilms, isolated matrix, cell wall, and cell cytoplasm by scintillation counting. Assays were performed in triplicate for each *Candida* isolate. Consistent with previous findings in *C. albicans* (13, 19) the majority of H^3-fluconazole is present in the extracellular matrix for each of these species, with very little or no drug found intracellularly or in the cell wall (Fig. 1B).

We next determined the effect of matrix β-1,3 glucan hydrolysis on biofilm susceptibility to fluconazole. Using a 96-well plate format, biofilm cell metabolic activity was assayed following exposure to fluconazole and β-1,3 glucanase alone and in combination using a tetrazolium salt XTT [2,3-bis-(2-methoxy-4-nitro-5-sulfophenyl)-2H-tetrazolium-5-carboxanilide inner salt] reduction assay (20, 21, 22, 23). Briefly, after 24 h biofilm growth, media was replaced by fresh RPMI-MOPS with dilutions of fluconazole at 1mg/mL, β-1,3 glucanase (Zymolyase - 20T, MP Biomedicals) at 0.7 U/mL, or a combination of the two. The β-1,3 glucanase concentration was chosen based upon our previous studies with *C. albicans* demonstrating synergy with fluconazole and no effect on cell viability for the enzyme alone (12, 19). Experiments were performed in triplicate. Drug effect is expressed at the percent biofilm reduction relative to growth of untreated controls. The statistical significance of differences among therapies was determined using analysis of variance (ANOVA). Similar to previous reports, fluconazole alone exhibited minimal activity against biofilms for each strain and species (12, 19). The low concentration of β-1,3 glucanase also produced little change in cell metabolic activity. However, fluconazole caused marked biofilm reduction in the presence of the β-1,3 glucan hydrolyzing enzyme. This effect was observed for all strains tested (Fig. 1C).

The results of the present study with non-albicans *Candida* species are similar to those from *C. albicans,* which demonstrate the contribution of biofilm matrix β-1,3 glucan for the antifungal drug resistance phenomenon linked to this common infection lifestyle (12, 13, 14). The relative impact of the mechanism for these other common *Candida* species appear congruent with that shown for *C. albicans* based upon comparable concentrations of matrix β-1,3 glucan,

antifungal drug sequestration, and influence of β-1,3 glucanase treatment on fluconazole efficacy. The prevalence of these non-*albicans* *Candida* species continues to rise. Insight to the mechanisms responsible for resistance to therapy is critical for design of new treatment strategies. The present study suggests that drug development targeting matrix β-1,3 glucan may potentiate the activity of currently available antifungal options.

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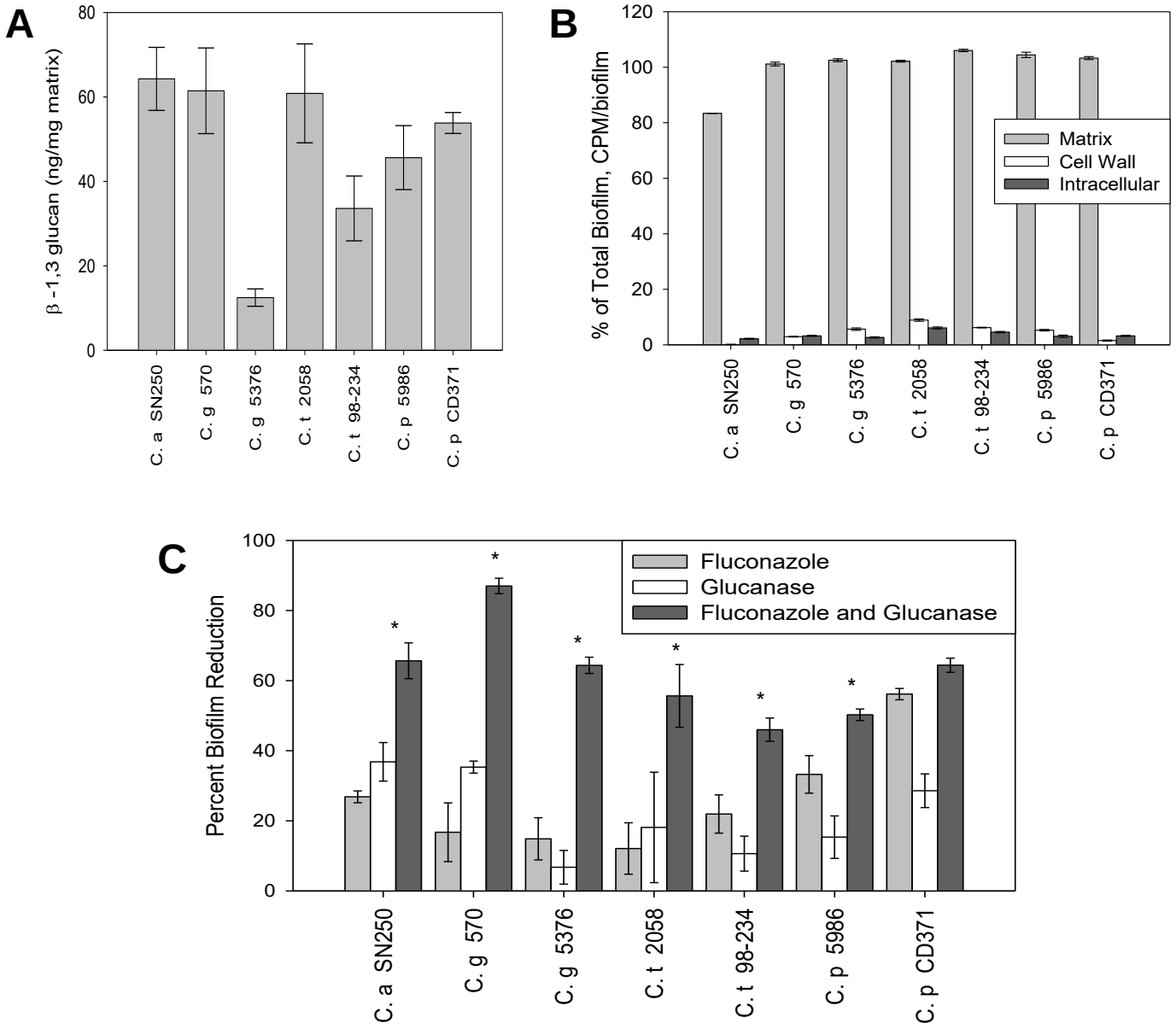
Figure Legends:

Figure 1A: The amount of β-1,3 glucan in a 1mg/ml sample of purified in vitro biofilm matrix, measured by ELISA. Samples are measured in triplicate. Standard Errors are shown.

Figure 1B: [H^3] fluconazole in the matrix, cell wall, and cytoplasm of in vitro biofilms. Shown as a percentage of [H^3] fluconazole in the total biofilm, measured as CPM/biofilm. Standard Errors are shown.

Figure 1C: Biofilm susceptibility to fluconazole and/or β-1,3 glucanase as measured by the XTT reduction assay. Fluconazole dosed at 1mg/ml and β-1,3 glucanase dosed at 0.7 U/ml. Data shown are representative examples, each read in triplicate. * Denotes a p value of <0.05 when comparing the combined drug values to either of the single drug values using a One-Way ANOVA.

Figure 1


A
β -1,3 glucan (ng/mg matrix)
0
20
40
60
80
C. a SN250
C. g 570
C. g 5376
C. t 2058
C. t 98-234
C. p 5986
C. p CD371
B
% of Total Biofilm, CPM/biofilm
0
20
40
60
80
100
120
Matrix
Cell Wall
Intracellular
C. a SN250
C. g 570
C. g 5376
C. t 2058
C. t 98-234
C. p 5986
C. p CD371
C
Percent Biofilm Reduction
0
20
40
60
80
100
Fluconazole
Glucanase
Fluconazole and Glucanase
*
C. a SN250
C. g 570
C. g 5376
C. t 2058
C. t 98-234
C. p 5986
C. p CD371

Chapter 3: Role of matrix polysaccharides in antifungal resistance of non-*albicans Candida* biofilms

Section 2: Mannan and β-1,6 glucan

Following the work in Chapter 3 Section 1, the role of two other extracellular matrix polysaccharides, α-mannan and β-1,6 glucan, in the drug resistance of clinically relevant *Candida* species was examined. The strains used were *C. parapsilosis* CLiB214 (a laboratory strain), *C. glabrata* 5376 (clinical isolate), and *C. tropicalis* 2058 (clinical isolate). The *C. albicans* laboratory strain SN250 was included for comparison (shown as Ref in Figure 1).

Enzyme and pharmacological inhibitors were used to inhibit matrix α-mannan and β-1,6 glucan. As mentioned in Chapter 2, mannan accumulation can be blocked with tunicamycin (TM), an antibiotic that inhibits N-glycosylation, and α-mannosidase, an enzyme that catalyzes the hydrolysis of terminal mannosides [1]. Brefeldin A (BFA), an inhibitor of anterograde transport between the ER and Golgi, was used to impair matrix deposition of both mannan and β-1,6 glucan, as are synthesized in the secretory pathway [2].

Biofilms were treated with fluconazole, one of the inhibitors, or a combination of fluconazole and inhibitor. 1000 μg/ml fluconazole was used to treat all biofilms except *C. parapsilosis* CLiB214, for which 250 μg/ml was used as this species exhibits slightly lower levels of drug resistance than *C. albicans.*

For experiments with TM (1.0 μg/ml) and BFA (0.6 μg/ml) (Sigma), biofilms were formed for 6 h prior to a 24 h dose. Biofilms treated with α-mannosidase (0.78 U/ml) (Jack Bean, Sigma) were formed for 24 h before a 24 h dose. All biofilms were formed in 96-well polystyrene plates in RPMI-MOPS. Experiments with α-mannosidase used RPMI pH 4.5 for the treatment period, the pH at which the enzyme is active. Following treatments, biofilm metabolic activity was read using the XTT assay [3].

For all combinations of fluconazole and inhibitor, each strain exhibited greater biofilm reduction compared to biofilms treated with only fluconazole or only inhibitor. This suggests that inhibiting matrix α-mannan or β-1,6 glucan potentiates fluconazole activity on *Candida* biofilms.

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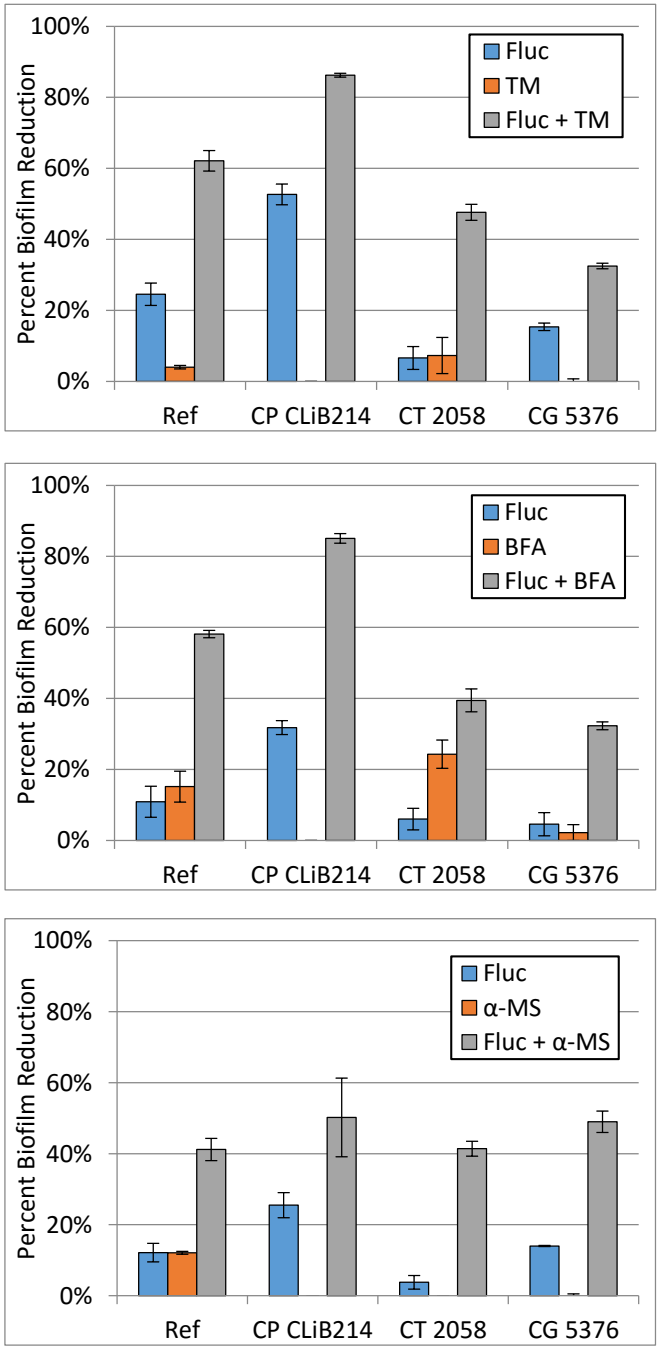
Figure 1. *Candida* biofilms were treated with fluconazole (Fluc) alone or in combination with tunicamycin (TM), Brefeldin A (BFA), or α-mannosidase (α-MS). The percent reduction in biofilm formation is shown, calculated using untreated biofilms for comparison. Standard errors are shown.



Chapter 4

Characterization of Extracellular Vesicle Production by *Candida albicans* Biofilms

The contents of this section will be submitted for publication pending ongoing analyses (outlined in Chapter 5): **Kaitlin F. Mitchell**, Robert Zarnowski, Hiram Sanchez, Stephen Plesh, Anna Jaromin, Jörg Bernhardt, and David R. Andes.

K. Mitchell performed SEM, developed extracellular vesicle (EV) quantification methods, collected EV samples, and performed all mutant strain characterization. R. Zarnowski created mutant strains, developed protocols for EV collection, performed 'omics' analyses, and established relationships with collaborators. H. Sanchez grew many of the large-scale biofilms used for EV collection, and performed measurements of matrix carbohydrates and protein. S. Plesh grew biofilms for EV collection and assisted with initial antifungal susceptibility testing of mutants. A. Jaromin is a collaborator from the University of Wroclaw, Poland, and performed TEM and sizing analysis. J. Bernhardt is a collaborator from Ernst-Moritz-Arndt University, Germany, and assisted with proteomic analysis.

INTRODUCTION

Biofilms are the preferred mode of growth for many microorganisms, both in the environment and as infection in a host. A hallmark of biofilms is production of an extracellular matrix, which provides protection and stability to the community. In an infection setting, this corresponds to inhibition of drugs, making biofilms incredibly difficult to eradicate [1, 2]. Better understanding of biofilm matrix composition, production, and secretion will provide new avenues for treating these infections.

Our group recently defined the biochemical composition of the matrix produced by *Candida albicans* biofilms [3]. This fungal pathogen tends to form highly drug-resistant biofilms on implanted medical devices, with cells dispersed from the biofilm leading to highly lethal disseminated disease [4, 5]. The matrix composition was found to be a complex mixture of all four organic molecules, with a specific carbohydrate complex implicated in binding antifungals [3, 6]. Another intriguing facet of this investigation came from the lipid analysis, which identified sterol esters and phospholipids in addition to free fatty acids. While the presence of some cell membrane in the matrix would be expected due to occasional lysis of biofilm cells, we wondered if these lipids might have an additional form or purpose.

One mechanism for extracellular transport of large molecules involves extracellular vesicles, or EVs. Variants of EVs have been identified in cell types across biological domains, with diverse functions including unconventional secretion of macromolecules, waste management, and intercellular signaling [7, 8]. EVs have been identified in multiple species of fungi, and a few reports have suggested the presence of EVs within biofilms, but no there has been no direct evidence for the role of EVs in transport of biofilm matrix [9-12]. Here, we report that *C. albicans* biofilms produce EVs, and that these structures are important for the accumulation of functional matrix.

RESULTS

Identification of extracellular vesicles in *Candida albicans* biofilms

In attempt to visualize extracellular vesicles from *C.albicans* biofilms, we used several methods to image both purified samples and intact biofilms (**Figure 1**). Purified EVs were analyzed using scanning and transmission electron microscopy, and proton correlation spectroscopy was employed to determine the average particle size of 200 nm diameter. Both imaging techniques revealed highly concentrated circular structures, with the size range of 10-300 nm diameter congruent with those measured with our sizing analysis. We then attempted to identify similar structures in intact biofilms using high-magnification SEM, and found these on the surface of both hyphal and yeast-form cells within the biofilm.

To quantify purified EVs we utilized imaging flow cytometry, which collects images of individual particles and can precisely measure particle concentration [13, 14]. Purified EV samples were stained with CFSE, a fluorescent dye that binds amine groups. To investigate the production of EVs over biofilm development, we quantified purified EVs from biofilms of different ages in time course experiments. We found that EV levels increase between 6 and 24 h, and that levels reach a sharp spike by 48 h and decline by 72 h.

Content of biofilm extracellular vesicles

We employed multiple techniques to investigate the biochemical composition of *C. albicans* biofilm EVs (**Figure 2**). We hypothesized that EVs are responsible, at least in part, for physically delivering materials to the extracellular matrix. These materials could include the carbohydrate, protein, lipid, and nucleic acid structural matrix components, as well as the enzymes thought to remodel them. Carbohydrates from purified EVs were analyzed using gas chromatography (GC) to measure monosaccharide abundance, revealing mannose to be the most abundant sugar (84.7% of all monosaccharides), followed by ribose (11.5%) and glucose (3.1%). As the predominant polysaccharides in the matrix are types of mannan and glucan, these

data suggest that EVs could be responsible for their export. GC was also used to identify lipid components, with the majority identified as diacylglycerols and phospholipids. The finding of phospholipids is not unexpected, as EVs in many systems are thought to be at least partially derived from the plasma membrane. The high proportion of DAGs, a key signaling molecule for cellular processes such as the protein kinase C pathway, could indicate that EVs export communication signals to either neighboring microbes, the host, or both.

Protein content of purified EV samples was analyzed using Hybrid Ion trap-Orbitrap mass spectrometry, revealing over 250 distinct proteins. There is a high level of overlap (77%) between the proteome of biofilm EVs and that previously reported for biofilm matrix [3], suggesting that many of the matrix proteins could be delivered to the extracellular space in vesicle packages. Protein functions within this group are diverse, with major biological processes including protein and carbohydrate metabolism, adhesion, and virulence. We used the same approach to analyze EVs derived from *C. albicans* yeast cultures, and compared relative abundance of the proteomes using a Voronoi treemap (**Figure 3**). The most upregulated proteins in biofilm EVs compared to yeast EVs are listed in **Table S1**.

Cellular components involved in extracellular vesicle production

As an approach to study biofilms with abnormal levels of EVs, we created mutants lacking gene products in several cellular pathways. We selected gene candidates that have previously been shown or predicted to impact EV production in fungi or other biological systems [15]. Our candidates included mostly gene products in the endosomal sorting complex required for transport (ESCRT), which are involved in formation of multivesicular bodies (MVBs) and sorting proteins for vacuolar degradation [15, 16]. Namely, these genes are *RIM20, VPS27, VPS4, HSE1, SNF7,* and *BRO1*. It has been demonstrated in other biological systems that some populations of EVs are derived from intraluminal vesicles formed in MVBs [8, 15]. We also selected *GRH1* for our candidate list of EV-production mutants, which is a homolog of the human Golgi reassembly

stacking protein (GRASP). Canonically, this enzyme is localized to the Golgi membrane, but has also been predicted to have a plasma membrane distribution [16-19]. Studies in several organisms have found that deleting GRASP does not affect conventional secretion or cisternal stacking, but instead inhibits the unconventional secretion of vesicles [17, 20-22]. In *Cryptococcus*, GRASP/Grh1p was found to be involved in secretion of the capsule polysaccharide glucuronoxylomannan (GXM), though not directly shown to do so by affecting EV secretion [23].

EVs from biofilms formed by each of these homozygous deletion mutants were quantified (**Figure 4**. All mutant biofilms produced fewer EVs compared to the reference strain SN250, according to both relative fluorescence and absolute particle counts. While all readings were lower than the reference EVs with both methods, measurements of absolute particle count were uniformly lower than a more dynamic range of readings for relative fluorescence. Since CFSE stains amine groups, this could indicate that the overall number of EVs is similar for all mutants, but that some have varied levels of protein or perhaps clump together to produce a brighter overall signal. Regardless, these data suggest that these cellular components impact EV production in *C. albicans*, and that their roles in this are conserved across multiple eukaryotes. Several mutants were analyzed for their size distribution. One mutant, *grh1*Δ/Δ, had similar sizes of EVs present compared to the reference strain SN250. Interestingly, three other mutants (*vps4*Δ/Δ, *vps23*Δ/Δ, and *vps27*Δ/Δ) showed similar variations in EV sizing. This could indicate separate roles for these gene products in the production of different EV populations.

Extracellular vesicle mutants have altered matrix abundance and function

To test our hypothesis that EVs are responsible for physically delivering materials to the extracellular matrix more directly, we quantified the matrix from biofilms formed by the mutants with deficient EV production (**Figure 6**). All mutants had lower amounts of matrix than the reference strain SN250, though the overall biomass of these biofilms was comparable, ruling out an overall growth defect. We next measured quantities of matrix protein and carbohydrate, finding

reduced amounts of both in each of the mutants except *VPS4*, which had levels of protein similar to SN250. These experiments with mutant biofilms reveal a trend where biofilms lacking normal EVs have altered levels of both overall matrix and types of matrix components, and lend support to the proposed role of EVs in matrix delivery.

Given the alteration of extracellular matrix composition when EV levels are not normal, we wondered if EVs could also alter matrix function, namely drug resistance (**Figure 7**). We tested each of the EV production mutants for biofilm susceptibility to the antifungal fluconazole, and found that several of these mutants were significantly more susceptible to treatment than the reference strain: *vps4Δ/Δ, vps27Δ/Δ, snf7Δ/Δ,* and *hse1Δ/Δ*. This shows that some mutants with deficient levels of EVs also have deficient matrix, and therefore deficient resistance to antifungal treatment. Mutant phenotypes were confirmed by testing complementation strains for drug susceptibility: those with a copy of the gene introduced had restored resistance.

DISCUSSION

Extracellular vesicles have been identified across biological systems, and their roles in fundamental processes ranging from the transport of virulence factors to the delivery of nutrient-scavenging enzymes have been realized in recent years. Here we provide the first direct evidence for the importance of EVs in biofilms, the preferred mode of growth for many microorganisms. Our previous identification of lipids in the extracellular matrix of *Candida albicans* biofilms, combined with the growing literature on the role of EVs in transporting varied types of molecules, prompted us to question if EVs produced by *C. albicans* biofilms could be responsible for delivering matrix materials or their modifying enzymes to the extracellular space.

We successfully purified EVs from biofilm cultures following methods similar to those used in studies with other microbes [8, 24]. The presence and size of these, as well as EVs within intact biofilms, was analyzed using multiple methods (**Figure 1**). Utilizing imaging flow cytometry, we

were able to determine the concentration of EVs isolated throughout the stages of biofilm development.

We performed several large-scale biochemical analyses to define the composition and cargo of biofilm EVs. The monosaccharide content was found to contain predominantly mannose – the monomer of the extracellular matrix carbohydrate mannan. Our lipid analysis identified polar lipids and sterol esters, which likely correspond to the EV membranes, as well as a high proportion of diacylglycerol and free fatty acids, which could be present as signaling molecules within the vesicles. Consistent with previous reports, both DNA and RNA were identified as well [25-27]. Our proteomic analysis revealed a highly diverse population – nearly 300 distinct proteins – with a variety of functions.

To explore the possibility that EVs deliver protein components to the matrix, we compared the current proteome to our previously collected data for the extracellular matrix [3]. The high degree of overlap between these data supports this theory. Many of the biofilm EV proteins are involved in processes relevant to the extracellular matrix environment, such as carbohydrate modification and metabolism, stress response, and nutrient acquisition. We also considered that biofilm EVs contain proteins specific to growth in this lifestyle, and therefore collected proteomic data from *C. albicans* yeast cultures. The proteome we identified for EVs from *C. albicans* yeast is largely similar to previously published data [28, 29].

Little is known regarding the cellular pathways involved in extracellular vesicle production, particularly in microorganisms. Creation of deletion mutants lacking putative EV production components provided a way to discern these pathways, as well as a useful tool for understanding the impact of EVs on the biofilm. All tested mutant biofilms produced lower concentrations of EVs than the reference strain, and all produced lower levels of matrix, suggesting that EVs are responsible for normal accumulation of matrix. Several of these mutants were also markedly susceptible to the antifungal fluconazole. Together, these data suggest that EVs, and the matrix components they transport, are important for normal biofilm resistance. It is worth considering

that each of these mutants likely produces a distinct population of EVs, with variations in size as noted in **Figure 5** but also variations in protein cargo. Perhaps these differences could account for some mutants maintaining drug resistance while others do not. Further studies will include investigation of size distribution and cargo for all mutant EVs.

The identification of a mechanism responsible for delivery of numerous extracellular matrix materials represents a potent target for biofilm infections – a way to inhibit the multiple materials simultaneously. Our comparison of biofilm EVs to those from yeast cultures reveals distinct protein cargo under biofilm conditions, which could be used as specific markers for biofilm infection.

MATERIALS AND METHODS

Media. *Candida albicans* strains were kept in 25% (vol/vol) glycerol for storage at -80^{o}C, and maintained on YPD medium with uridine. All biofilms were grown in RPMI-MOPS media at 37^{o}C in rolling bottles as previously described [3], and inoculated from overnight yeast cultures grown in YPD at 30^{o}C. For mutant strain construction, transformants were selected on minimal medium with the appropriate auxotrophic supplements.

Strains and strain construction. The strains developed for this study are listed in **Table S2**. The parent strain SN152 was used for creation of homozygous deletion mutants using fusion PCR disruption cassettes as previously reported [6]. Complementation of mutant strains with a single gene copy used selection for arginine prototrophy [30]. Both fusion cassettes and plasmids for complementation were transformed in *C. albicans* cells using the lithium acetate method. Colony PCR was used to verify all genotypes.

Extracellular vesicle collection, sizing, and quantification. Concentration of culture supernatant and isolation of extracellular vesicles was adapted from previously published

methods [24]. Biofilms were grown in rolling bottles for 48 h, and planktonic cultures were grown in 2 L shaking flasks overnight. Fungal cells were removed from supernatents by passing through a 0.2 µm filter. The supernatant was concentrated to a volume roughly 25 ml using an Amicon ultrafiltration system (size cutoff, 30 kDa). To ensure removal of cellular debris, the supernatant was ultracentrifuged at 40,000 x g for 1 h and pellets discarded. The supernatents were then transferred to a new tube and spun at 100,000 x g for 1.5 h. The pellets were resuspended in 1x PBS and stored at 4^{o}C with 0.2% sodium azide added as preservative.

The average particle size of purified extracellular vesicles was determined using a Zetasizer Nano-ZS (Malvern Instruments). Particle diameter was analyzed using a photon correlation spectroscopy (PCS), following a volume distribution algorithm. All measurements were performed at 25^{o}C.

To quantify extracellular vesicles, samples were stained with carboxyfluorescein succinimidyl ester (CFSE) at final concentration 3.2 µM. Samples were allowed to incubate at 37^{o}C for 15 min prior to analysis. Relative fluorescence was measured on an automated plate reader at excitation/emission 485/515 nm. Absolute particle quantification was performed on an imaging flow cytometer (ImageStream Mark II, Amnis). Gating strategy is described in **Figure S1**.

Imaging purified extracellular vesicles and biofilms. For SEM of purified EVs, the sample was allowed to adhere to a sterile coverslip placed in a 12-well plate overnight at 4^{o}C prior to fixation. For SEM of biofilms, 40 ul of an inoculum of 10^{8} cells/ml in RPMI-MOPS was added to the coverslips and incubated 60 min at 37^{o}C. 1 ml RPMI-MOPS was added to each well and the plates were incubated at 37^{o}C for 20 h, shaking at 50 rpm. 1 ml fixative (4% formaldehyde, 1% glutaraldehyde in PBS) was then added to each well prior to incubation at 4^{o}C overnight. Coverslips were then washed with PBS prior to incubation for 30 min in 1% osmium tetroxide at room temperature. Samples were then serially dehydrated in ethanol (30 to 100%). Critical point

drying was used to completely dehydrate the samples prior to palladium-gold coating. Samples were imaged on a SEM LEO 1530, with Adobe Photoshop 7.0.1 used for image compilation.

TEM of purified EVs was performed using previously described methods [6, 24]. Samples were fixed in 4% formaldehyde and 2% glutaraldehyde, then treated with 1% osmium tetroxide and 1% potassium ferricyanide. Samples were then stained with 1% uranyl acetate and dehydrated in a series of ethanol washes. They were embedded in Spurr's resin, placed on copper grids and then poststained with 8% uranyl acetate in 50% methanol and Reynolds' lead citrate. Samples were imaged using a Philips CM 120 TEM. NIH Image J software was used to approximate vesicle diameter.

Compositional analysis of extracellular vesicles. Purified extracellular vesicles were trypsin digested for analysis by nanoLC-MS/MS (Agilent 1100 Nanoflow), connected to a hybrid linear ino trap-orbitrap mass spectrometer (LTQ-Orbitrap) equipped with a nanoelectrospray ion source [3]. Raw MS/MS data were searched using an in-house MASCOT search engine for *C. albicans* amino acid sequence. Further annotation and functional mapping of identified peptides was performed using Voronoi Tree Map diagrams [31]. Lipid analysis was performed as described [3]. Lipids were extracted from the purified EV sample using 2:1 $CHCl_3$ and MeOH and analyzed by gas chromatography. Matrix monosaccharide composition and quantification was performed on alditol acetate derivatives by GLC-FIC (Shimadzu GC-2010 system) as previously reported [3].

Extracellular matrix analysis. For quantification of biofilm and extracellular matrix dry mass, biofilms were scraped from bottles after 48 h and gently sonicated to remove matrix material from fungal cells [3]. This mixture was then centrifuged to separate a pellet of cells from a matrix supernatant. The matrix supernatant and the resuspended pellet were both frozen and lyophilized, allowing for measurements of dry mass.

For subsequent extracellular matrix analysis, the dry material was resuspended in PBS and aliquoted for protein and carbohydrate measurements. Samples of extracellular matrix were compared to a wildtype sample collected in parallel. Carbohydrates were quantified colorimetrically at 492 nm using the phenol-sulfuric method [32]. Proteins were quantified colorimetrically at 562 nm using the BCA protein assay (Pierce Biotechnology).

Biofilm drug susceptibility testing. An inoculum of 10^6 cells per ml in RPMI-MOPS was made for each strain, with 100 µl used in 96-well plates. After a 6 h biofilm formation period at 37°C, media and non-adherent cells were removed. Fresh media and dilutions of fluconazole (250 to 1000 µg/ml) were added for 48 h incubation with an additional drug and media exchange at 24 h.

Biofilms were quantified using a tetrazolium salt XTT (2,3-bis-(2-methoxy-4-nitro-5-sulfophenyl)-2H-tetrazolium-5-carboxanilide inner salt)) reduction assay [33, 34]. 90 µl XTT (0.75 mg/ml) and 10 µl phenazine methosulfate (PMS) (320 µg/ml) were added for 30 min at 37°C, and an automated plate reader was used to measure absorbance at 492 nm. Biofilm reduction was calculated by comparing untreated control biofilms to those with treatment.

Acknowledgements

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Figure Legends

Fig. 1. SEM was used to image extracellular vesicles purified from biofilm culture (A), as well as those within an intact biofilm both on the surface of hyphal (B) and yeast form (C) cells. Purified vesicles were also imaged using TEM (D). (E) Size distribution of extracellular vesicles shows the average particle diameter to be 200 nm. (F) Production of extracellular vesicles over biofilm development, quantified using imaging flow cytometry. Data are shown as particles per milliliter of purified vesicles stored in PBS following purification.

Fig. 2. Carbohydrates and lipids were analyzed for using gas chromatography (GC). Values represent percentages of the entire carbohydrate or lipids in the purified extracellular vesicle sample.

Fig. 3. Comparison of protein content between extracellular vesicles from biofilm or yeast cultures, and visualized with Voronoi treemap. Relative protein abundance is shown with a heat map increasing from blue to red, and grouped by predicted cellular process.

Fig. 4. Extracellular vesicles purified from mutant biofilms were fluorescently stained with CFSE and quantified using an automated plate reader (A) or imaging flow cytometry (B).

Fig. 5. Size distribution of vesicles purified from mutant biofilms, showing the range of diameter measurements (nm). Peaks represent the proportion of the total EV sample corresponding to the diameter on the x-axis. Large peaks (>2000 nm diameter) likely represent cell debris or protein aggregates in the sample.

Fig. 6. The extracellular matrix from vesicle-deficient biofilms was characterized. (A) Dry weight of purified matrix, shown with overall biomass of the biofilm for comparison. Raw measurements

were in mg, and are shown as percentage of the wildtype strain SN250. (B) Protein and carbohydrate composition of the mutant matrices, compared to SN250.

Fig. 7. Fluconazole susceptibility of mutants deficient in extracellular vesicle production (A) and complemented strains with one gene copy added back (B). Shown as the percent of biofilm reduction following drug treatment, calculated by comparison to untreated biofilms for each strain. Biofilms were quantified using the XTT assay. Standard errors are shown.

Supporting Information Legends

Fig. S1. Gating strategy for imaging flow cytometry with extracellular vesicles stained by CFSE. Internal calibration beads were first gated out based on their high auto fluorescence in the side scatter channel (designated Ch06). The “not beads” group was then analyzed for brightfield signal (Ch01) by area and aspect ratio, using the conservative rationale that large dye clumps are visible in this channel and the smaller EVs are not. CFSE-positive particles (Ch02) that fit these criteria were quantified as particles per milliliter of sample.

Figure 1

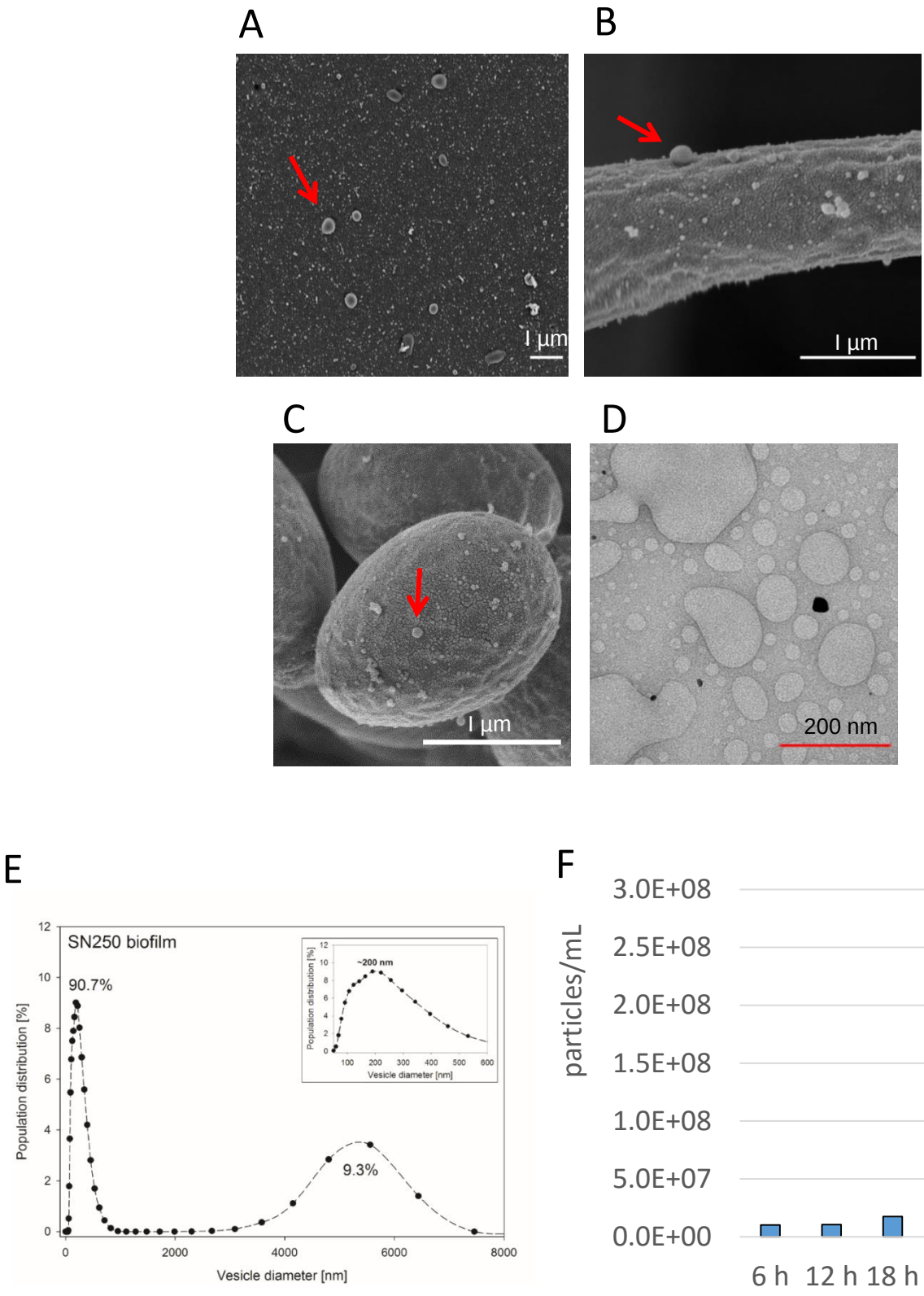
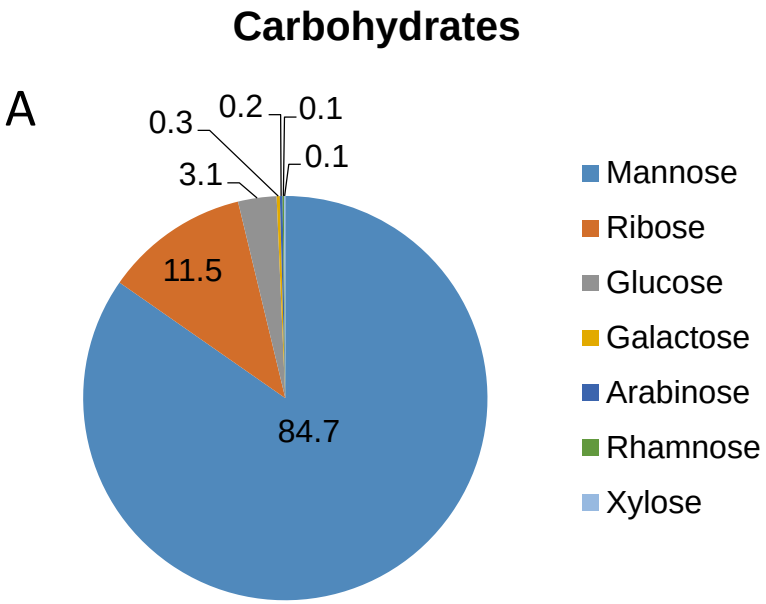

A
B
1 µm
1 µm
C
D
1 µm
200 nm
E
SN250 biofilm
90.7%
9.3%
~200 nm
Population distribution [%]
Vesicle diameter [nm]
F
particles/mL
3.0E+08
2.5E+08
2.0E+08
1.5E+08
1.0E+08
5.0E+07
0.0E+00
6 h
12 h
18 h
24 h
48 h
72 h

Figure 2


A
Carbohydrates
0.3
0.2
0.1
0.1
3.1
11.5
84.7
Mannose
Ribose
Glucose
Galactose
Arabinose
Rhamnose
Xylose

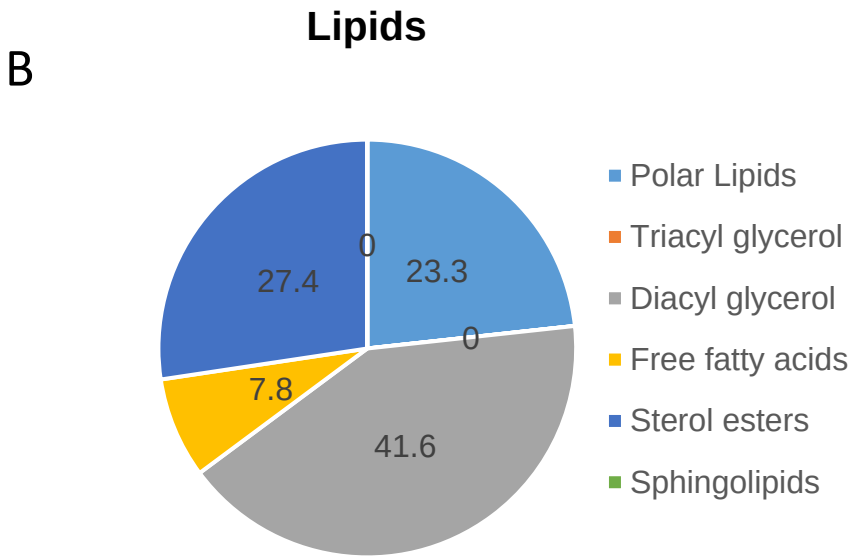

B
Lipids
0
23.3
27.4
0
7.8
41.6
Polar Lipids
Triacyl glycerol
Diacyl glycerol
Free fatty acids
Sterol esters
Sphingolipids

Figure 3

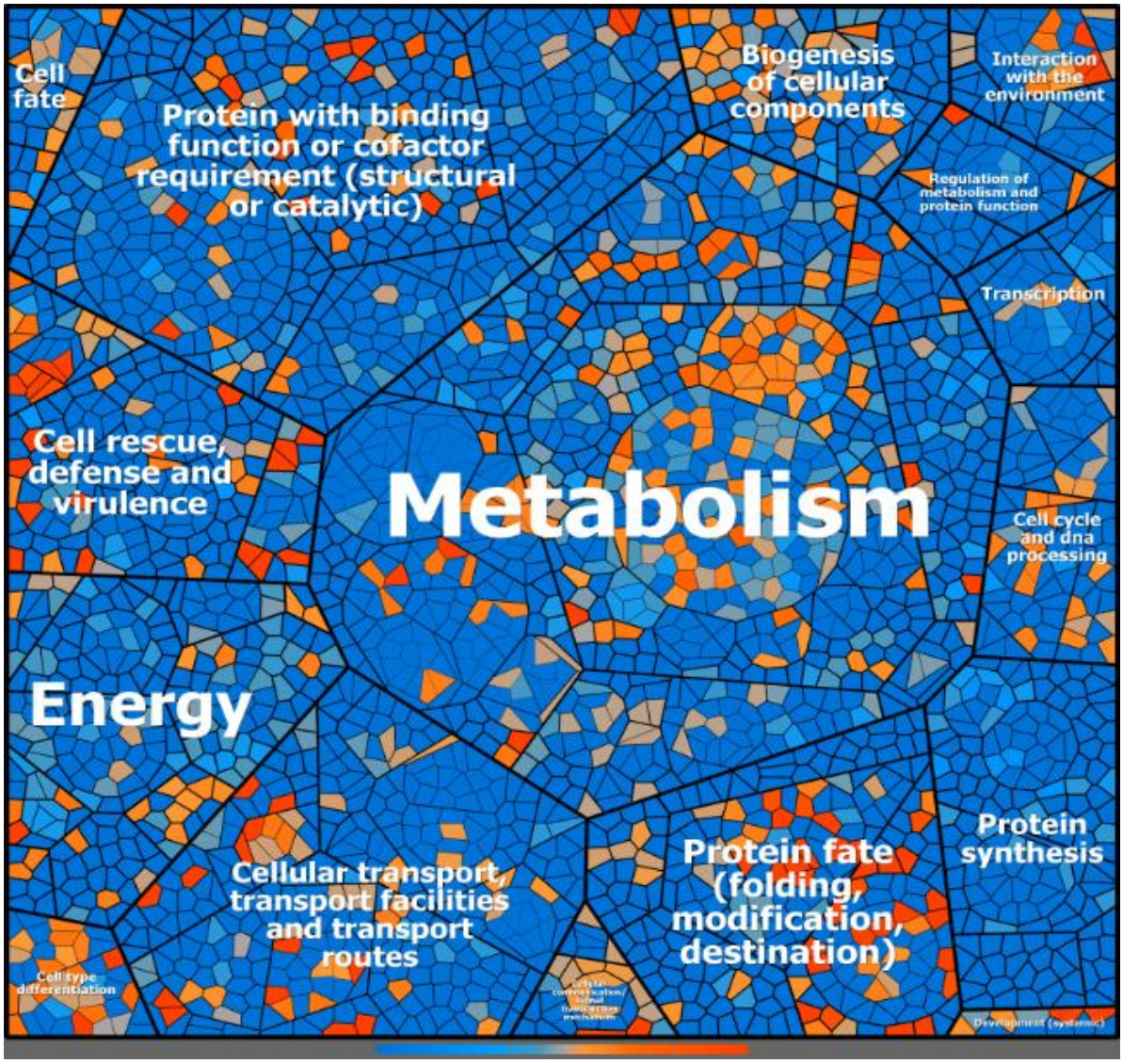
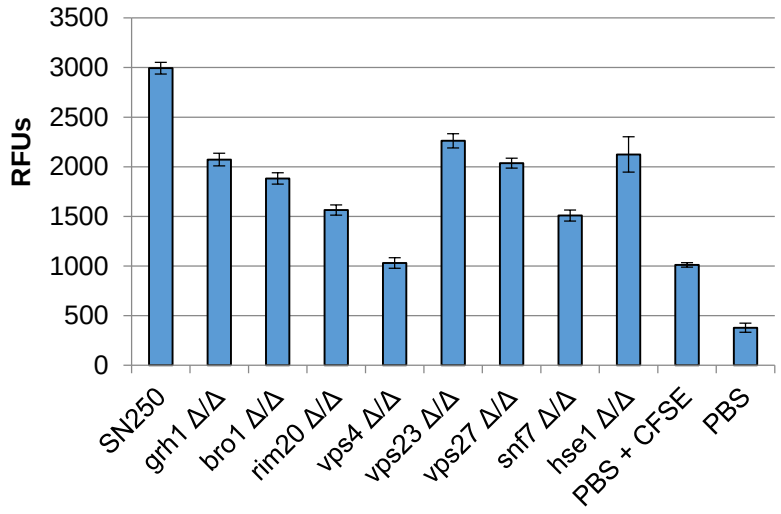

Cell fate
Protein with binding function or cofactor requirement (structural or catalytic)
Biogenesis of cellular components
Interaction with the environment
Regulation of metabolism and protein function
Transcription
Cell rescue, defense and virulence
Metabolism
Cell cycle and dna processing
Energy
Protein synthesis
Cellular transport, transport facilities and transport routes
Protein fate (folding, modification, destination)
Cell type differentiation

Figure 4

A


3500
3000
2500
2000
1500
1000
500
0
RFUs
SN250
grh1 Δ/Δ
bro1 Δ/Δ
rim20 Δ/Δ
vps4 Δ/Δ
vps23 Δ/Δ
vps27 Δ/Δ
snf7 Δ/Δ
hse1 Δ/Δ
PBS + CFSE
PBS

B

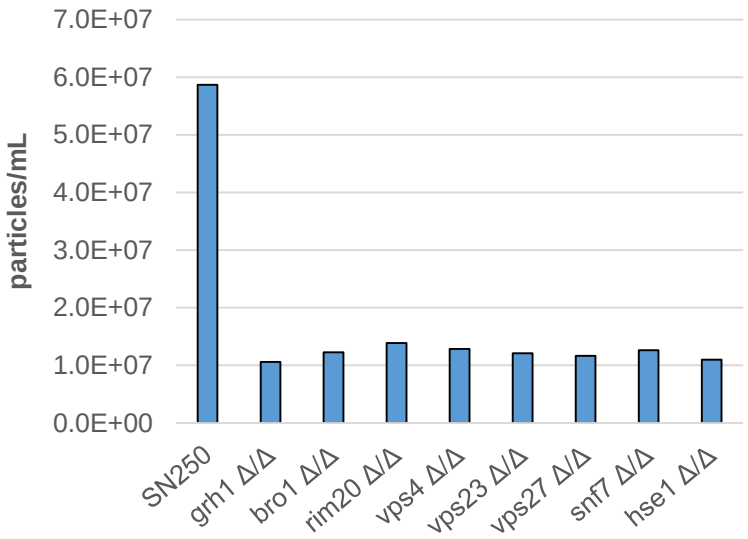
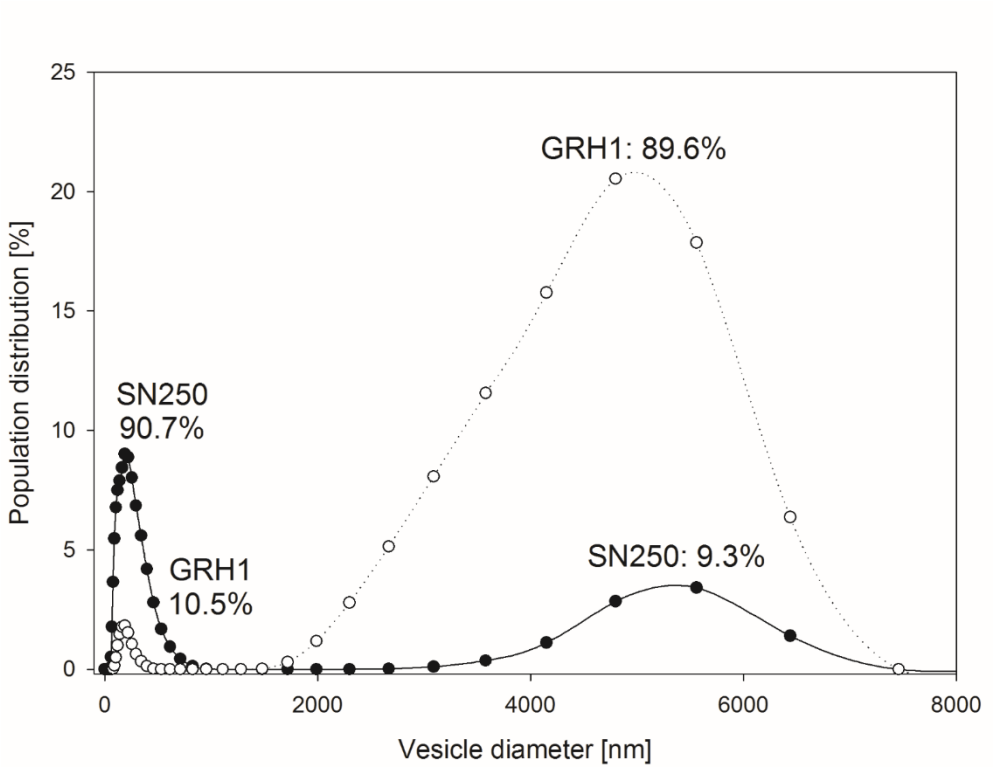

7.0E+07
6.0E+07
5.0E+07
4.0E+07
3.0E+07
2.0E+07
1.0E+07
0.0E+00
particles/mL
SN250
grh1 Δ/Δ
bro1 Δ/Δ
rim20 Δ/Δ
vps4 Δ/Δ
vps23 Δ/Δ
vps27 Δ/Δ
snf7 Δ/Δ
hse1 Δ/Δ

Figure 5

A


GRH1: 89.6%
SN250
90.7%
GRH1
10.5%
SN250: 9.3%
Population distribution [%]
Vesicle diameter [nm]
0
5
10
15
20
25
0
2000
4000
6000
8000

B

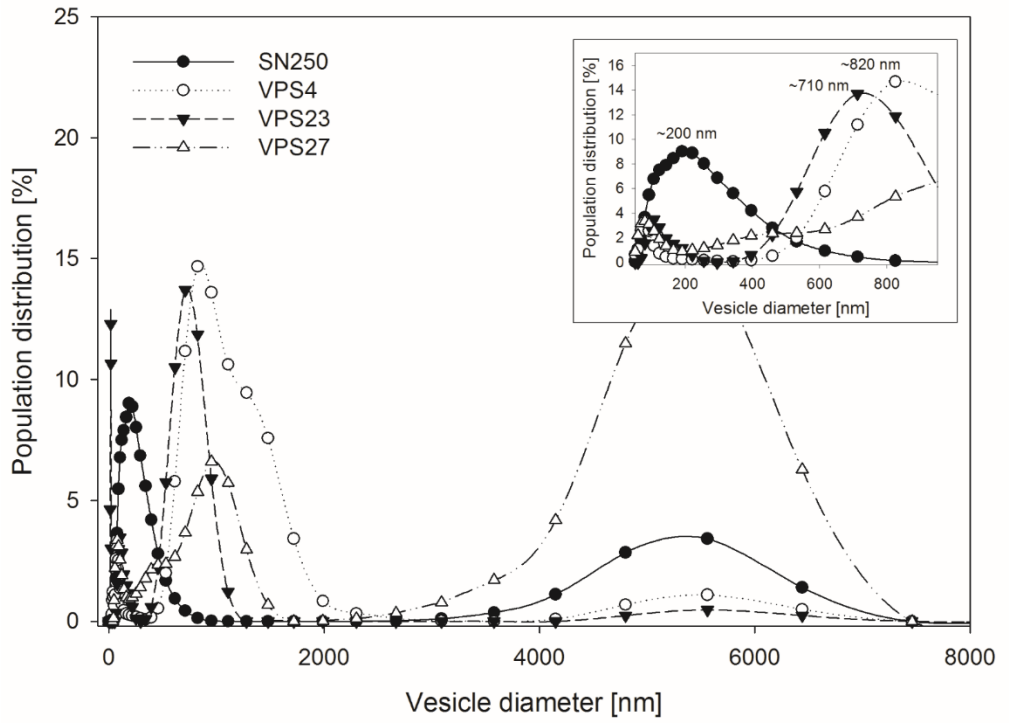
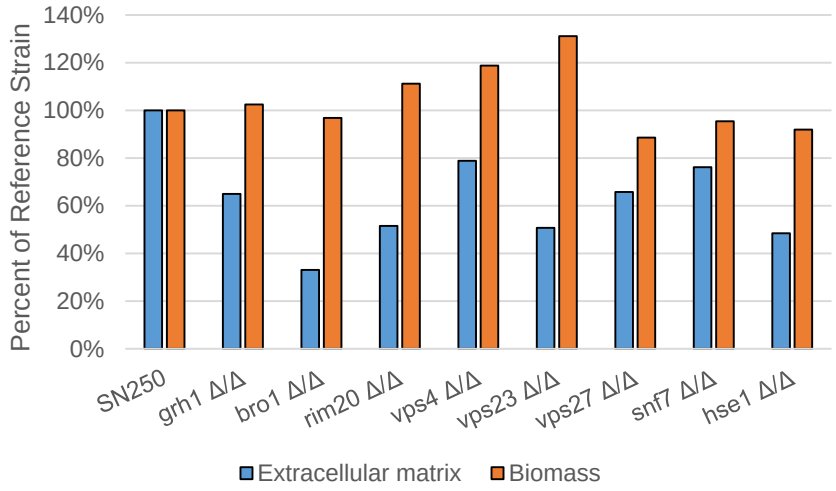

SN250
VPS4
VPS23
VPS27
~200 nm
~710 nm
~820 nm
Population distribution [%]
Vesicle diameter [nm]
Population distribution [%]
Vesicle diameter [nm]

Figure 6

A


Percent of Reference Strain
140%
120%
100%
80%
60%
40%
20%
0%
SN250
grh1 Δ/Δ
bro1 Δ/Δ
rim20 Δ/Δ
vps4 Δ/Δ
vps23 Δ/Δ
vps27 Δ/Δ
snf7 Δ/Δ
hse1 Δ/Δ
Extracellular matrix
Biomass

B

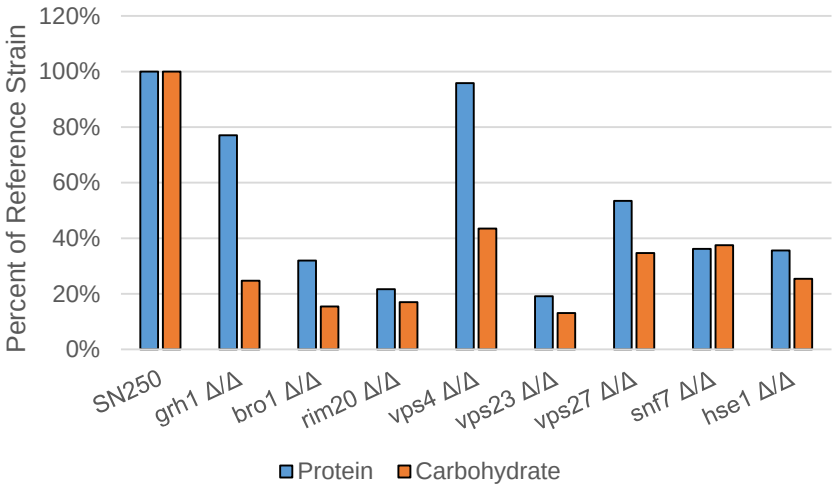
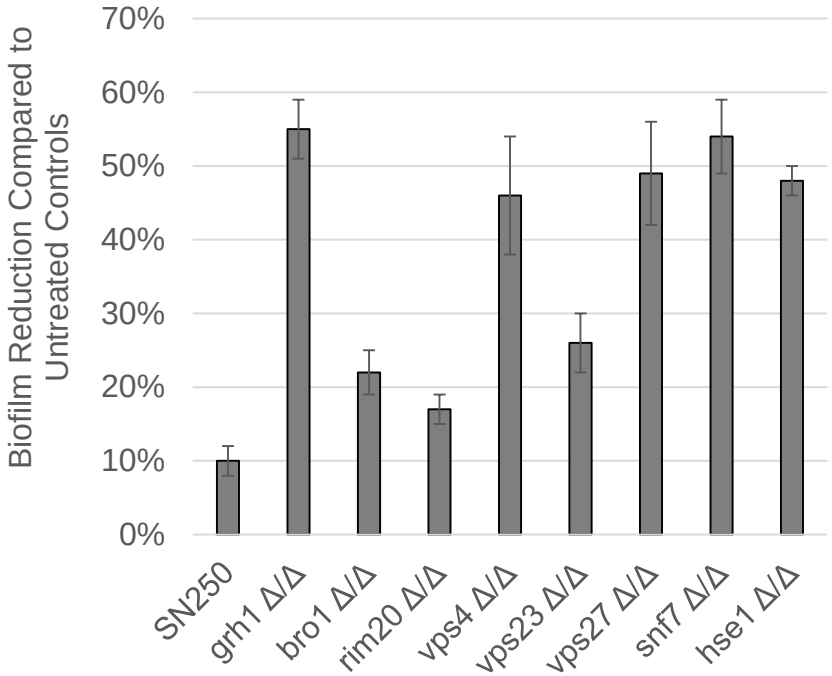

Percent of Reference Strain
120%
100%
80%
60%
40%
20%
0%
SN250
grh1 Δ/Δ
bro1 Δ/Δ
rim20 Δ/Δ
vps4 Δ/Δ
vps23 Δ/Δ
vps27 Δ/Δ
snf7 Δ/Δ
hse1 Δ/Δ
Protein
Carbohydrate

Figure 7

A


Biofilm Reduction Compared to Untreated Controls
70%
60%
50%
40%
30%
20%
10%
0%
SN250
grh1 Δ/Δ
bro1 Δ/Δ
rim20 Δ/Δ
vps4 Δ/Δ
vps23 Δ/Δ
vps27 Δ/Δ
snf7 Δ/Δ
hse1 Δ/Δ

B

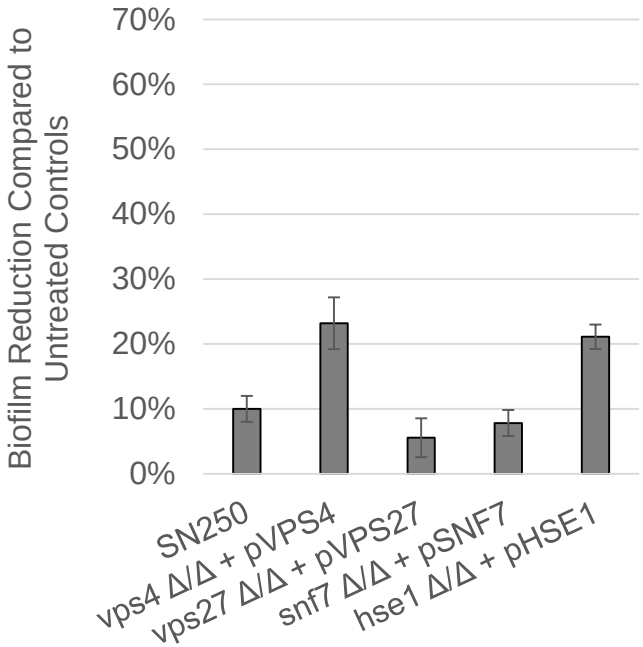
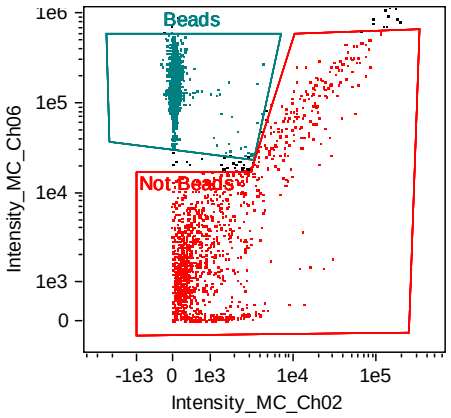
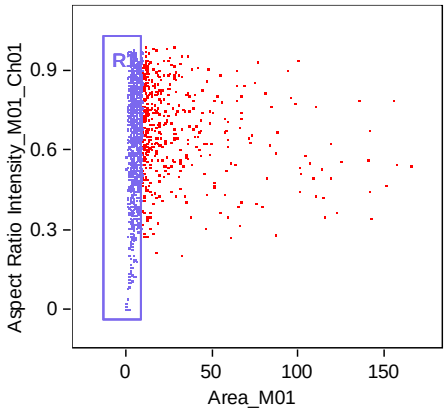

Biofilm Reduction Compared to Untreated Controls
70%
60%
50%
40%
30%
20%
10%
0%
SN250
vps4 Δ/Δ + pVPS4
vps27 Δ/Δ + pVPS27
snf7 Δ/Δ + pSNF7
hse1 Δ/Δ + pHSE1

Figure S1

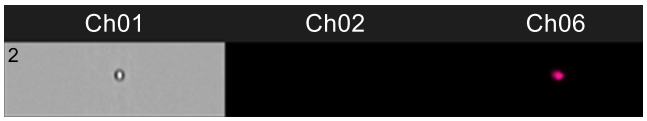


Not beads



Beads

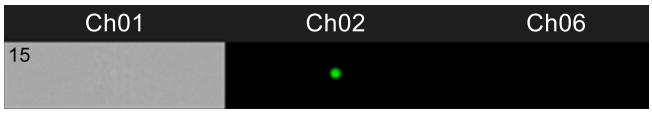
High brightfield, high side scatter



Not Beads

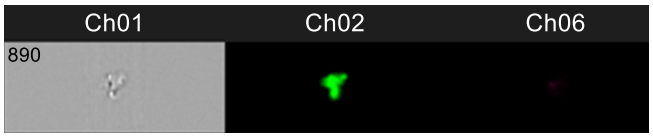
EVs: gate R1

Minimal brightfield



Not EVs

High brightfield



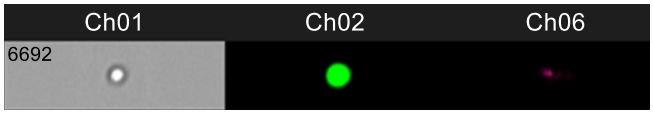


Table S1. Extracellular Vesicle Proteins Upregulated in Biofilms

Gene Name	ORF	Biofilm*	Yeast*	Ratio**	GO Function
SAP5	orf19.5585	330.4	2.0	165.62	hydrolase, peptidase
PRA1	orf19.3111	479.5	4.0	120.18	hydrolase, protein binding, peptidase
MGP1	orf19.1239	245.8	4.0	61.60	molecular function
HYR1	orf19.4975	78.6	2.0	39.39	protein binding
AMS1	orf19.2768	52.4	2.0	26.26	hydrolase
OP4	orf19.4934	100.7	4.0	25.25	molecular function
ECE1	orf19.3374	74.5	3.3	22.42	molecular function
ASM3	orf19.6037	64.5	3.3	19.39	hydrolase
UTR2	orf19.1671	10.1	0.7	15.15	hydrolase, transferase
HEX1	orf19.6673	42.3	3.3	12.72	hydrolase
PHR1	orf19.3829	98.7	10.0	9.90	hydrolase, transferase
SUR7	orf19.3414	12.1	1.3	9.09	molecular function
CSH1	orf19.4477	22.2	2.7	8.33	oxidoreductase
SUN41	orf19.3642	145.1	23.3	6.23	hydrolase
LPD1	orf19.6127	12.1	2.0	6.06	oxidoreductase
PRE2	orf19.2233	8.1	1.3	6.06	hydrolase, peptidase
orf19.2777	orf19.2777	54.4	10.6	5.11	not yet annotated
CHT2	orf19.3895	42.3	8.6	4.89	hydrolase
PRE8	orf19.7335	6.0	1.3	4.54	hydrolase, peptidase
TOS1	orf19.1690	118.9	27.9	4.26	molecular function
ATC1	orf19.6214	8.1	2.0	4.04	hydrolase
PGA4	orf19.4035	26.2	6.7	3.94	transferase
GCA1	orf19.4899	80.6	21.3	3.79	hydrolase
MP65	orf19.1779	139.0	42.6	3.27	hydrolase
NCE102	orf19.5960	8.1	2.7	3.03	molecular function
PUP3	orf19.1336	4.0	1.3	3.03	hydrolase, peptidase, enzyme regulator
POX1-3	orf19.1652	28.2	10.0	2.83	oxidoreductase
PGM2	orf19.2841	12.1	4.7	2.60	isomerase activity
CTR1	orf19.3646	6.0	2.7	2.27	transporter activity
RBE1	orf19.7218	6.0	2.7	2.27	lipid binding
YPT1	orf19.3052	4.0	2.0	2.02	hydrolase, protein binding
CRH11	orf19.2706	8.1	5.3	1.51	hydrolase, transferase
POR1	orf19.1042	16.1	11.3	1.43	transporter activity
ENG1	orf19.3066	34.3	24.6	1.39	hydrolase
GDH3	orf19.4716	6.0	4.7	1.30	oxidoreductase
SAP6	orf19.5542	24.2	0.0	0.0	hydrolase, peptidase

C2_09800C_A	orf19.1370	22.2	0.0	0.0	molecular function
CDR1	orf19.6000	14.1	0.0	0.0	hydrolase, transporter
IFD7	orf19.1048	14.1	0.0	0.0	oxidoreductase
C5_02230W_A	orf19.4230	12.1	0.0	0.0	hydrolase, peptidase
CSA2	orf19.3117	12.1	0.0	0.0	molecular function
LIP4	orf19.2133	10.1	0.0	0.0	hydrolase
YPS7	orf19.6481	10.1	0.0	0.0	hydrolase, peptidase
SOD5	orf19.2060	10.1	0.0	0.0	oxidoreductase
CR_06500C_A	orf19.716	8.1	0.0	0.0	molecular function
PBR1	orf19.6274	8.1	0.0	0.0	molecular function
CR_09240C_A	orf19.7322	6.0	0.0	0.0	molecular function
KEX2	orf19.4755	4.0	0.0	0.0	hydrolase, peptidase, enzyme regulator
PRE10	orf19.6582	4.0	0.0	0.0	hydrolase, rna binding, peptidase
RAX2	orf19.3765	4.0	0.0	0.0	molecular function

*Biofilm and Yeast values represent the number of peptides detected by MS/MS in the entire EV protein sample, normalized to the total amount of protein

**Ratio values represent the ratio of Biofilm to Yeast peptides

Table S2. Mutant Strains Used in this Study

Gene Name	Systematic Name	Genotype	Strain Name	Description*
GRH1	orf19.71	Δ/Δ	URZ407	Ortholog(s) have role in ER to Golgi vesicle-mediated transport, protein secretion and COPII vesicle coat, Golgi membrane, cis-Golgi network localization
BRO1	orf19.1670	Δ/Δ	URZ370	role in transport from multivesicular body to vacuole; not involved in Rim101 pathway; macrophage and pseudohyphal-repressed; flow model biofilm induced; interacts with SNF7; has homolog Rim20
RIM20	orf19.4800	Δ/Δ	URZ361	homolog to BRO1; Protein involved in the pH response pathway; binds to the transcription factor Rim101 and may serve as a scaffold to facilitate the C-terminal proteolytic cleavage that activates Rim101; required for alkaline pH-induced hyphal growth
VPS4	orf19.4339	Δ/Δ	URZ368	Involved in transport from MVB to the vacuole and ESCRT-III complex disassembly; mutation decreases SAP secretion and virulence in murine intravenous infection; regulated by Gcn2p, Gcn4p; required for normal Rim8p processing
VPS23	orf19.2343	Δ/Δ	URZ364	Involved in proteolytic activation of Rim101, which regulates pH response
VPS27	orf19.6031	Δ/Δ	URZ362	Endosomal protein that forms a complex with Hse1p; required for recycling Golgi proteins, forming lumenal membranes and sorting ubiquitinated proteins destined for degradation; has Ubiquitin Interaction Motifs which bind ubiquitin
SNF7	orf19.6040	Δ/Δ	URZ366	Role in proteolytic activation of Rim101 and Rim8 processing/activation; separable roles in RIM101 pathway and in transport from MVB to vacuole; involved in echinocandin and azole sensitivity; interacts with BRO1
HSE1	orf19.3233	Δ/Δ	URZ358	Subunit of the endosomal Vps27p-Hse1p complex; complex is required for sorting of ubiquitinated membrane proteins into intralumenal vesicles prior to vacuolar degradation, as well as for recycling of Golgi proteins and formation of lumenal membranes

*Based upon *Candida* or *Saccharomyces* Genome Database

Chapter 5

Conclusions and Future Directions

Genetic determinants of matrix polysaccharide synthesis

A study published in 2014 by the Andes group identified the three major polysaccharide components of the *Candida albicans* biofilm extracellular matrix: α-Mannan, β-1-6 glucan, and β-1,3 glucan [1]. The work described here (Chapter 2) identified the cellular components responsible for the production of these matrix materials. The creation of over 50 mutant strains, each lacking a gene in the synthesis pathways for these polysaccharides, was a critical tool for this project.

Biofilms for each mutant were assayed for normal matrix structure, composition, and function. A group of seven genes were found to govern levels of matrix mannan (*ALG11, MNN9, MNN11, VAN1, MNN4-4, PMR1, VRG4*), and two genes govern levels of matrix β-1,6 glucan (*BIG1, KRE5*). The β-1,3 glucan synthase, *FKS1*, was also investigated.

Interactions of matrix polysaccharides

Using these mutants, as well as pharmacologic and enzymatic methods, to inhibit these polysaccharides, it was determined that α-mannan, β-1-6 glucan, and β-1,3 glucan are each required for normal assembly and function of the matrix. Inhibition of one polysaccharide resulted in lower matrix levels of the others, and led to increased biofilm susceptibility to multiple classes of antifungals. Pull-down experiments using monoclonal antibodies for each polysaccharide supported the notion that these molecules physically interact.

To explore the nature of how these polysaccharides might be assembled, experiments using mixed mutant biofilms were then performed. Biofilms were inoculated with mutants from each of the different polysaccharide synthesis pathways in equal proportion, then assayed as before for matrix composition and function. For example, a mutant lacking a mannosyltransferase gene was grown in a biofilm with a β-1,6 glucan synthase mutant. We found that these mixed biofilms did have restored levels of overall matrix, and in some cases had much greater amounts of matrix than either wildtype biofilms or each mutant biofilm individually.

These experiments, while simple, demonstrate something fundamental about biofilm matrix assembly: it must occur extracellularly. If these components were assembled within the cell, for instance, during polysaccharide extension in the Golgi, simply mixing the two different mutants would not yield normal matrix, because the mannan created by the β-1,6 glucan mutant would never physically contact the β-1,6 glucan from the mannan mutant.

Matrix Polysaccharides: Future Directions

There are several unanswered questions for this project. First, what is the nature of the interactions between α-mannan, β-1-6 glucan, and β-1,3 glucan? They could very likely be covalently connected by a protein intermediate. In a pull down study for β-1,3 glucan, an uncharacterized protein was detected and termed *MGP1* (Major Glucan Protein 1) (Robert Zarnowski, unpublished). It would be interesting to look for proteins that co-precipitate with the other two, more abundant, polysaccharides.

An interesting trend was observed when measuring matrix components in the mixed mutant biofilms: levels of α-mannan, β-1-6 glucan, and β-1,3 glucan were each higher in all the mixed biofilms compared to the wildtype or single mutant biofilms. One possible explanation for this could be the existence of a signaling feedback mechanism. If the biofilm cells are able to sense an abnormal matrix, perhaps they upregulate their own matrix production pathways. This could be tested by measuring expression of polysaccharide synthesis genes in the mixed mutant biofilms.

In attempt to establish the ‘functional complementation’ of the matrix observed in mixed mutant biofilms, purified matrix from one mutant was added to another from a different polysaccharide production pathway. However, these results from these tests were inconclusively negative – under the conditions tested, no change in drug susceptibility occurred. This could be because the assembly of matrix materials is sensitive to time as well as the specific concentrations of those materials. However, regardless of experimental parameters, it is possible that adding

purified matrix to biofilms is not a feasible approach. Matrix assembly might be sufficiently intricate that mass amounts of matrix added exogenously cannot be incorporated.

Finally, an ongoing effort in the Andes group is the investigation of these matrix production pathways in non-*albicans Candida* species that are clinically relevant. Work in Chapter 3 implicated the matrix carbohydrates in providing normal biofilm drug resistance. The role of the genes investigated in Chapter 2 will be explored in other species, to see if their role in matrix accumulation and function is conserved.

Candida albicans Biofilm Extracellular Vesicles

The work in Chapter 4 describes the discovery and characterization of extracellular vesicles (EVs) produced by *C. albicans* biofilms. The protein cargo of these EVs were found to have a high degree of overlap with proteins in the biofilm matrix, suggesting that EVs could be a delivery system for matrix components to the extracellular space. Mutants were created that lack genes proposed to be involved in EV synthesis; deletion of these resulted in lower levels of EV production by biofilms, as well as lower levels of matrix produced by these biofilms. For several mutants, increased susceptibility to antifungals was also observed. Together, these data have indicated EVs are a mechanism for delivering matrix materials, and are thus accountable for inherent biofilm traits attributable to the matrix.

Extracellular Vesicles: Future Directions

There are several ongoing experiments for this project. First, the presence and type of nucleic acid will be investigated, as it was in a study with *Paracoccidioides spp*. EVs [2]. Purified EVs were found to stain positively for Sytox Green, a fluorescent DNA marker (Hiram Sanchez, unpublished), but the concentration and sequence will be further explored. Additionally, the presence of specific polysaccharide components will be queried by using monoclonal antibodies for α-mannan, β-1-6 glucan, and β-1,3 glucan in and ELISA with purified EVs. If these are

detected, it could suggest that EVs are used transport the MGCx to the matrix. Also in progress is a more detailed lipid analysis. Robert Zarnowski has established a collaboration with the Kansas State Lipidomics Research Center to obtain detailed measurements of different lipid classes including phospholipids and sphingolipids.

Another ongoing experiment involves the addition of purified extracellular vesicles to mutant biofilms. Previous data suggested that this could restore the drug susceptibility of a vesicle-deficient mutant, such as *grh1*Δ/Δ. However, that data has proved inconclusive, as multiple repeated trials have not yielded the same result. Also, since that data was obtained, this mutant strain was rechecked and found to be incorrect. Adding purified EVs to a correct mutant with a strong drug susceptibility phenotype has not had an effect.

As an alternative approach to test for a possible interaction between antifungals and extracellular vesicles, 1H-NMR is being employed. This technique was previously used by Robert Zarnowski to observe a decrease in the proton signal output for fluconazole as purified matrix was added [1]. A similar approach will be used by adding purified EVs to fluconazole.

The work presented here has helped to improve our understanding of biofilms at a basic biological level, but with important clinical implications. It is my hope that these findings will be expanded upon in other biological systems, especially those where the extracellular matrix hinders effective management of biofilm infections.

1. Zarnowski, R., et al., *Novel entries in a fungal biofilm matrix encyclopedia.* MBio, 2014. **5**(4): p. e01333-14.
2. Peres da Silva, R., et al., *Extracellular vesicle-mediated export of fungal RNA.* Sci Rep, 2015. **5**: p. 7763.

Appendix A: Impact of extracellular vesicle cargo on *Candida albicans* biofilms

	Peptide Abundance			Phenotypic Analysis of Deletion Mutants				
Gene	**Biofilm**	**Yeast**	**Ratio**	**Biofilm Reduction with Fluconazole***	**1 h Biofilm Adhesion (% WT)**	**48 h Biofilm Dispersion (% WT)**	**48 h Biofilm Formation (% WT)**	**Function**
REF	--	--	--	10-20%	100%	100%	100%	Strain SN250
SAP5	330.4	2	165.62	60%	60%	670%	45%	Secreted aspartyl proteinase
PRA1	479.5	4	120.18	16%	119%	122%	97%	Cell surface protein that sequesters zinc
ALS3	68.5	0.7	103.01	13%	137%	120%	101%	Cell wall adhesin; epithelial adhesion, endothelial invasion
MGP1	245.8	4	61.6	n/a	n/a	n/a	n/a	(Major Glucan Protein) Secreted protein
AMS1	52.4	2	26.26	50%	75%	127%	101%	Putative alpha-mannosidase
ASM3	64.5	3.3	19.4	6%	113%	124%	95%	Putative secreted acid sphingomyelin phosphodiesterase
UTR2	10.1	0.7	15.15	8%	140%	108%	95%	Putative GPI anchored cell wall glycosidase; role in adhesion
PHR1	98.7	10	9.9	60%	123%	277%	75%	Cell surface glycosidase
SUR7	12.1	1.3	9.09	10%	180%	147%	140%	Protein required for normal cell wall, plasma membrane, cytoskeletal organization, endocytosis; localizes to eisosome subdomains of plasma membrane
KRE9	6	0.7	9.09	10%	127%	97%	91%	B-1,6 glucan biosynthesis
CSH1	22.2	2.7	8.33	8%	109%	110%	94%	Aldo-keto reductase; role in fibronectin adhesion, cell surface hydrophobicity
SUN41	145.1	23.3	6.23	61%	204%	248%	115%	Cell wall glycosidase; role in biofilm formation and cell separation; possibly secreted; hypoxia, hyphal induced
PGA4	26.2	6.7	3.94	25%	140%	107%	95%	GPI-anchored cell surface protein; beta-1,3-glucanosyltransferase
GCA1	80.6	21.3	3.79	n/a	n/a	n/a	n/a	Glucoamylase
MP65	139	42.6	3.27	17%	173%	145%	88%	Cell surface mannoprotein; cell-wall glucan metabolism, adhesion; adhesin motif
NCE102	8.1	2.7	3.03	6%	154%	109%	112%	Non classical protein export protein
ERV25	4	1.3	3.03	51%	118%	131%	83%	Component of COPII-coated vesicles
XOG1	46.3	20.6	2.25	40%	76%	117%	92%	B-1,3 glucanase

CRH11	8.1	5.3	1.51	15%	144%	92%	103%	GPI-anchored cell wall transglycosylase
ENG1	34.3	24.6	1.39	5%	100%	104%	87%	Endo-1,3-beta-glucanase
EXG2	16	0	0	29%	147%	134%	108%	exo-1,3-beta-glucosidase
SAP6	24.2	0	0	24%	127%	106%	103%	Secreted aspartyl protease; expressed during hyphal growth, oral carriage, infection
ALS1	20.1	0	0	24%	96%	100%	90%	Cell-surface adhesin; adhesion, virulence, immunoprotective roles
PRE4	12.1	0	0	n/a	n/a	n/a	n/a	20S proteasome subunit
ZRT2	8.1	0	0	30%	97%	150%	92%	Zinc transporter

Peptide Abundance: Proteomic data of wildtype extracellular vesicles presented Chapter 4. Represents the abundance of these proteins in purified EVs from biofilms, yeast cells, or the ratio of biofilm to yeast.

Phenotypic Analysis of Deletion Mutants: Homozygous deletion mutants were created for each of the genes listed in the table. Mutants were screened for phenotypes relevant to biofilm formation and phenotypes potentially relevant to EV deficiencies: drug susceptibility and biofilm cell dispersion. Biofilms were grown and quantified following standard protocols described in previous Chapters.

Biofilm Reduction with Fluconazole: The reduction in biofilm formed after treatment with 1000 µg/ml Fluconazole, compared to untreated control biofilms.

1 h Biofilm Adhesion: The amount of biofilm adhered to polystyrene after 1 h, quantified by XTT. Shown as percentages compared to the reference strain.

48 h Biofilm Dispersion: The amount of cells dispersed from the biofilm after growth for 48 h. Free-floating cells were removed from the media of 96-well biofilms, placed in a fresh plate, and read for OD 600. Shown as percentages compared to the reference strain.

48 h Biofilm Formation: Quantified by XTT.

Function: Putative gene functions taken from *Candida* Genome Database.

Appendix B: Abstracts for manuscripts published in collaboration

An abstract follows for the manuscript published in collaboration with A. Mitchell:

Desai JV, Bruno VM, Ganguly S, Stamper RJ, **Mitchell KF**, Solis N, Hill EM, Xu W, Filler SG, Andes DR, Fanning S, Lanni F, Mitchell AP. Regulatory Role of Glycerol in Candida albicans Biofilm Formation. MBio. 2013 Apr 9;4(2): e00637-12. PMCID: PMC3622937.

Biofilm formation by Candida albicans on medically implanted devices poses a significant clinical challenge. Here, we compared biofilm-associated gene expression in two clinical C. albicans isolates, SC5314 and WO-1, to identify shared gene regulatory responses that may be functionally relevant. Among the 62 genes most highly expressed in biofilms relative to planktonic (suspension-grown) cells, we were able to recover insertion mutations in 25 genes. Twenty mutants had altered biofilm-related properties, including cell substrate adherence, cell-cell signaling, and azole susceptibility. We focused on one of the most highly upregulated genes in our biofilm proles, RHR2, which specifies the glycerol biosynthetic enzyme glycerol-3-phosphatase. Glycerol is 5-fold-more abundant in biofilm cells than in planktonic cells, and an rhr2Δ/Δ strain accumulates 2-fold-less biofilm glycerol than does the wild type. Under in vitro conditions, the rhr2Δ/Δ mutant has reduced biofilm biomass and reduced adherence to silicone. The rhr2Δ/Δ mutant is also severely defective in biofilm formation in vivo in a rat catheter infection model. Expression profiling indicates that the rhr2Δ/Δ mutant has reduced expression of cell surface adhesin genes ALS1, ALS3, and HWP1, as well as many other biofilm-upregulated genes. Reduced adhesin expression may be the cause of the rhr2Δ/Δ mutant biofilm defect, because overexpression of ALS1, ALS3, or HWP1 restores biofilm formation ability to the mutant in vitro and in vivo. Our findings indicate that internal glycerol has a regulatory role in biofilm gene expression and that adhesin genes are among the main functional Rhr2-regulated genes.

An abstract follows for the manuscript published in collaboration with A. Johnson:

Nobile CJ, Fox EP, Hartooni N, **Mitchell KF**, Hnisz D, Andes DR, Kuchler K, Johnson AD. A Histone Deacetylase Complex Mediates Biofilm Dispersal and Drug Resistance in *Candida albicans*. MBio. 2014 Jun 10;5(3):e01201-14. PMCID: PMC4056552.

Biofilms are resilient, surface-associated communities of cells with specialized properties (e.g., resistance to drugs and mechanical forces) that are distinct from those of suspension (planktonic) cultures. Biofilm formation by the opportunistic human fungal pathogen Candida albicans is medically relevant because C. albicans infections are highly correlated with implanted medical devices, which provide efficient substrates for biofilm formation; moreover, biofilms are inherently resistant to antifungal drugs. Biofilms are also important for C. albicans to colonize diverse niches of the human host. Here, we describe four core members of a conserved histone deacetylase complex in C. albicans (Set3, Hos2, Snt1, and Sif2) and explore the effects of their mutation on biofilm formation. We find that these histone deacetylase complex members are needed for proper biofilm formation, including dispersal of cells from biofilms and multifactorial drug resistance. Our results underscore the importance of the physical properties of biofilms in contributing to drug resistance and dispersal and lay a foundation for new strategies to target biofilm dispersal as a potential antifungal intervention.