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Role of Fks1p and Matrix Glucan in *Candida albicans* Biofilm Resistance to an Echinocandin, Pyrimidine, and Polyene▽

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***Candida* infections frequently involve drug-resistant biofilm growth on device surfaces. Glucan synthase gene *FKS1* has been linked to triazole resistance in *Candida* biofilms. We tested the impact of *FKS1* modulation on susceptibility to additional antifungal classes. Reduction of *FKS1* expression rendered biofilms more susceptible to amphotericin B, anidulafungin, and flucytosine. Increased resistance to anidulafungin and amphotericin B was observed for biofilms overexpressing *FKS1*. These findings suggest that *Candida* biofilm glucan sequestration is a multidrug resistance mechanism.**

In hospital settings, *Candida* spp. often cause disease by adhering to the surface of a medical device and adapting to a biofilm lifestyle (7, 10). Biofilms consist of cells attached to a surface and embedded in a protective matrix produced by the organisms (5). *C. albicans* biofilm cells are phenotypically distinct, and their ability to survive exposure to high antifungal concentrations presents a serious therapeutic dilemma (1, 2, 11, 14, 19–21). Biofilm cells exhibit up to 1,000-fold-increased resistance relative to free-floating, or planktonic, cells (3, 9, 12, 18).

Glucan synthesis by Fks1p has been implicated in *C. albicans* biofilm resistance to the azole drug fluconazole (17). *FKS1* disruption was found to reduce manufacture and deposition of β-1,3-glucan in the biofilm matrix, resulting in susceptibility to fluconazole. The matrix glucan was shown to sequester the triazole, preventing it from reaching its target. The mechanism is biofilm specific and has been studied only for the triazoles.

The purpose of this study was to determine the role of *FKS1* in *C. albicans* biofilm resistance to other available antifungal drug classes. We chose to study three strains with differing expressions of *FKS1* and concomitant variations in matrix glucan. The strains included a heterozygous deletion mutant (*FKS1*/*fks1*Δ), an *FKS1* overexpression mutant (*TDH3-FKS1*) with one *FKS1* allele under the control of *TDH3* promoter and one allele intact, and a reference strain (4, 17). Finally, because *FKS1* is essential in *C. albicans*, a conditional *TET-FKS1* mutant was also included (22). The *TET-FKS1* strain has one allele deleted and one allele under the control of a tetracycline- or doxycycline-repressible promoter. An echinocandin (anidulafungin), flucytosine, and amphotericin B deoxycholate were selected for their different mechanisms of action.

For biofilm antifungal susceptibility testing, *C. albicans* biofilms were grown in 96-well polystyrene plates as previously described (16, 20). Wells were inoculated with 10^6 cells/ml in RPMI medium-MOPS (morpholinepropanesulfonic acid). After an adherence period (6 or 24 h), biofilms were washed with phosphate-buffered saline (PBS). Fresh media and antifungals were applied, and plates were incubated for an additional 24 h at 37°C. The concentration ranges included those above and below the planktonic MIC values and included 0.001 to 0.125 μg/ml anidulafungin, 0.03 to 8 μg/ml flucytosine, and 0.008 to 2 μg/ml amphotericin B deoxycholate (13). After 24 h of incubation at 30°C, an XTT [2,3-bis(2-methoxy-4-nitro-5-sulfo-phenyl)-2*H*-tetrazolium-5-carboxanilide] reduction assay was performed and endpoints were determined spectrophotometrically at 492 nm as a measure of cell metabolic activity (16, 20).

For the *FKS1*/*fks1*Δ strain, the *TDH3-FKS1* strain, and the reference strain, we measured the impact of antifungal wells compared to the no-drug control wells. The impact of doxycycline repression of *FKS1* on antifungal susceptibility during biofilm formation was similarly examined using the *TET-FKS1* strain with a doxycycline concentration range of 1 to 240 ng/ml in a 96-well checkerboard format. After adherence, biofilms were incubated in the presence of the doxycycline and antifungal in combination for 24 h prior to the XTT assay. For planktonic studies, MICs were determined two times in duplicate and measured visually using CLSI endpoints (15).

Anidulafungin was the most effective against parent *C. albicans* biofilms, while flucytosine had minimal or no activity at the highest concentration tested (Fig. 1). The biofilm formed by the *FKS1*/*fks1*Δ heterozygote was more susceptible to flucytosine and anidulafungin, with drug impact at 2- to 8-fold-lower concentrations. Heterozygous *FKS1* disruption did not impact amphotericin B activity in this assay design. To determine if a difference for amphotericin B might be due to the phase of growth, a later phase of biofilm growth (24 h) was tested. By this method, *FKS1*/*fks1*Δ biofilms were more susceptible to amphotericin B than reference strain biofilms were, but the difference was less than that observed for the other antifungal drug classes (not shown). For example, treatment with amphotericin B at 0.25 μg/ml decreased *FKS1*/*fks1*Δ biofilms by 80%, compared to 60% for the reference strain ($P < 0.05$; Student's *t* test).

The *TET-FKS1* strain recapitulated the phenotypes for

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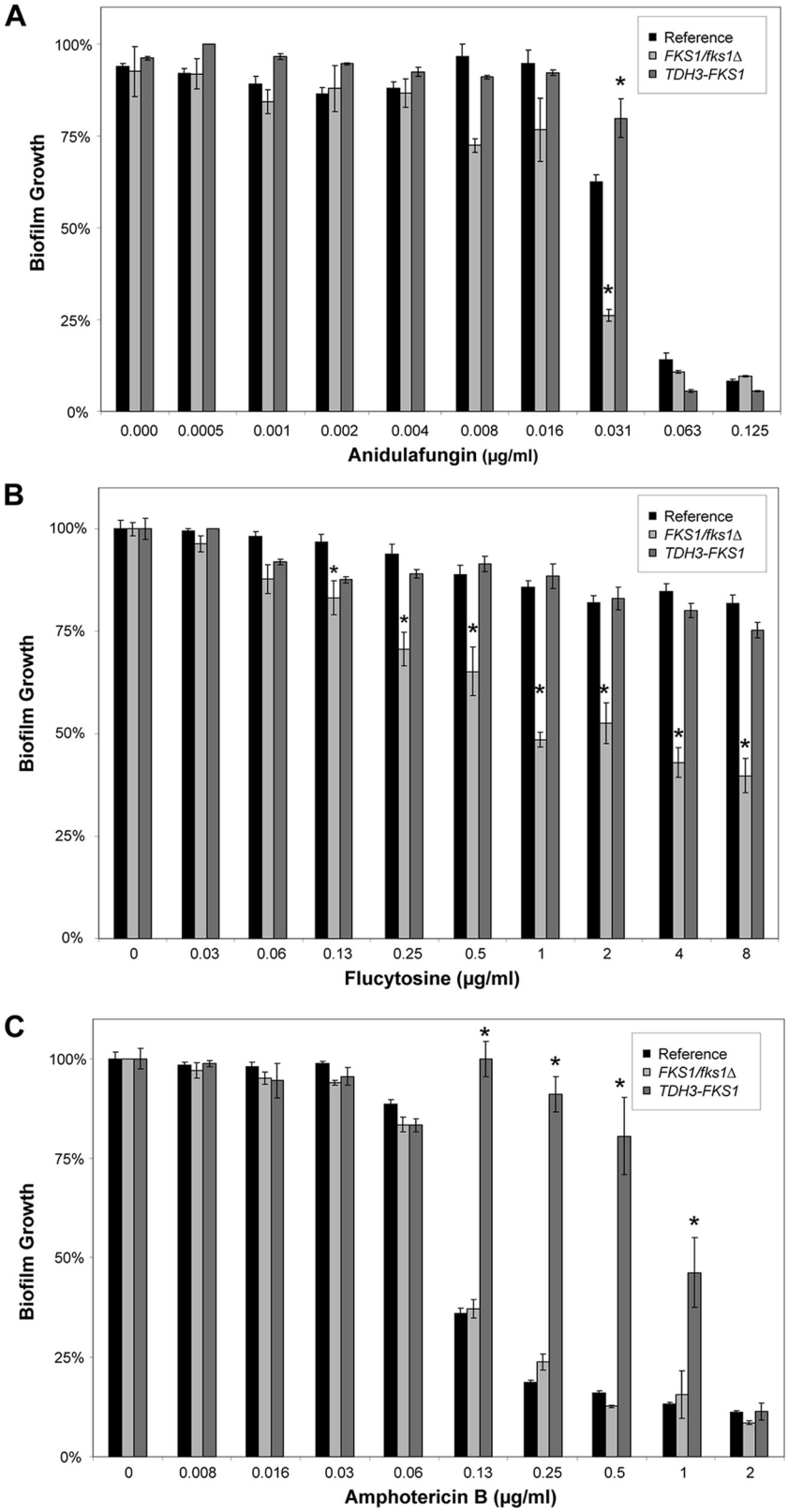

FIG. 1. Impact of *FKS1* modulation on antifungal susceptibility in *C. albicans* biofilms. *FKS1*-modulated biofilms were grown in 96-well plates for 6 h and treated with serial dilutions of anidulafungin (A), flucytosine (B), or amphotericin B deoxycholate (C) for an additional 24 h. Endpoints were assessed using an XTT assay, and data are shown as percentages of biofilm growth relative to growth of untreated controls. Assays were performed in triplicate, and each error bar represents one standard error. Statistical significance was determined by analysis of variance with pairwise comparisons using the Holm-Sidak method. *, $P < 0.05$.

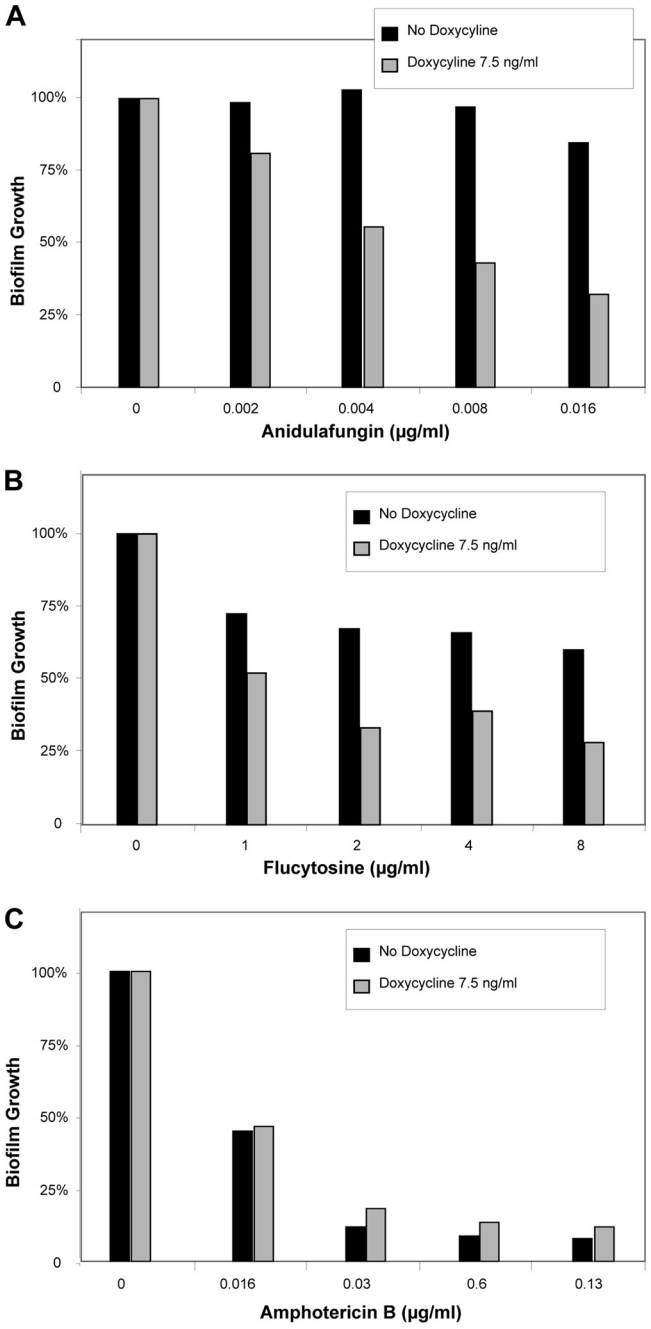

FIG. 2. Impact of doxycycline repression of *FKS1* on antifungal susceptibility in *C. albicans* biofilms. *TET-FKS1*-modulated biofilms were grown in 96-well plates for 6 h and treated for 24 h with serial dilutions of anidulafungin (A), flucytosine (B), or amphotericin B deoxycholate (C) in combination with doxycycline by using a checkerboard format. Endpoints were assessed using an XTT assay, and data are shown as percentages of biofilm growth relative to growth of untreated controls. Checkerboard assays were performed in duplicate, and results from one assay replicate are shown.

susceptibility to echinocandin and flucytosine, with 4- to 8-fold-lower drug concentrations effective for the condition with doxycycline repression of *FKS1* (Fig. 2). Interestingly, modulation of *FKS1* by doxycycline or heterozygous disruption did not render biofilms more susceptible to amphotericin B. The explanation for this difference is not clear. Doxycycline did not impact *C. albicans* reference strain growth or drug susceptibility at the concentrations used in these experiments (data not shown).

TABLE 1. Impact of *FKS1* modulation on drug susceptibility of planktonic cells[a]

Drug	MIC (μg/ml) for strain type		
	Reference strain	*FKS1*/*fks1*Δ	*TDH3-FKS1*
Anidulafungin	0.01	0.01	0.01
Flucytosine	0.06	0.03	0.03
Amphotericin B deoxycholate	0.03	0.03	0.03

[a] MICs were determined using the CLSI method and endpoints.

FKS1 overexpression had a similar but lesser impact on biofilm susceptibility to anidulafungin (Fig. 1). Increased resistance to flucytosine was not detectable by these overexpression assays, due to the profound resistance of the reference biofilm at the highest concentrations. The *TDH3-FKS1* overexpression biofilm exhibited a marked increased resistance to amphotericin B, supporting a role for glucan in polyene biofilm resistance.

Importantly, modulation of *FKS1* did not impact planktonic susceptibility to the various antifungals based on standard CLSI testing and interpretation (Table 1) (15). Because the drug target of anidulafungin is Fks1p, we considered the possibility that genetically modifying expression and regulation of this gene may directly impact susceptibility to the compound (6). For example, echinocandin resistance in planktonic cells has been linked to altered Fks1p kinetics due to point mutations in several hot spots (8). However, the *FKS1* heterozygote was similarly susceptible to echinocandin in this planktonic assay, while the strain was more susceptible to echinocandin in the biofilm assay relative to the parent strain, again suggesting a biofilm-specific mode of action (Table 1).

FKS1 has been linked to *C. albicans* resistance through a mechanism specific to biofilms. Investigations using fluconazole and amphotericin B suggest that this process involves antifungal sequestration by the matrix glucan (16, 17, 23). Modulation of *FKS1*, through either inhibition or overexpression, impacted biofilm susceptibility to all the antifungal agents tested. As observed for *FKS1* and fluconazole resistance, this mechanism appears to be biofilm specific, since disruption of *FKS1* has no impact on planktonic resistance. Our findings indicate that *FKS1* similarly impacts biofilm resistance to other antifungal drug classes, possibly through the same mechanism.

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