Genetic Basis of *Candida* Biofilm Resistance Due to Drug-Sequestering Matrix Glucan

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Medical devices provide an ecological niche for microbes to flourish as a biofilm community, protected from antimicrobials and host defenses. Biofilms formed by *Candida albicans*, the most common fungal pathogen, survive exposure to extraordinarily high drug concentrations. Here, we show that β-glucan synthase Fks1p produces glucan, which is deposited in the biofilm matrix. The extracellular glucan is required for biofilm resistance and acts by sequestering antifungals, rendering cells resistant to their action. These findings provide the genetic basis for how biofilm matrix production governs drug resistance by impeding drug diffusion and also identify a useful biofilm drug target.

Candida species are the fourth most frequent cause of bloodstream infections in hospitalized patients [1]. Their ability to grow as biofilms on medical devices is in part responsible for the escalating disease prevalence. Despite a growing antifungal armamentarium, there remains no effective medical treatment of this disease because of the profound resistance associated with biofilm growth. The discovery that *Candida* biofilm cells assemble an extracellular matrix composed of β-glucan led us to investigate and identify a contribution of this material to biofilm drug resistance [2].

The current studies examine of the role of β-1,3 glucan synthase gene *FKS1/GSC1* in triazole drug susceptibility, matrix production, and drug sequestration during *C. albicans* biofilm growth. Fks1p uses uridylyltransferase (UDP)–glucose to synthesize cell wall β-1,3 glucan during planktonic growth [3]. By examining strains with modulated *FKS1* expression, we found that β-1,3 glucan synthesis and deposition in the matrix is necessary for biofilm drug resistance, but is not needed for planktonic cell resistance. This carbohydrate traps the azole drug, fluconazole, preventing its intracellular action.

Methods. Animals were maintained in accordance with the University of Wisconsin animal care guidelines. Yeast strains were stored in 15% glycerol at −80°C and maintained on yeast extract (1%)-peptone (2%)-dextrose (2%) medium, with uridine at a concentration of 80 μg/mL, prior to experiments.

Strains were constructed from BWP17 (genotype *ura3::*λ*imm434/ura3::*λ*imm434*, *arg4::hisG/arg4::hisG*, *his1::hisG/his1::hisG*) and reference strain DAY185 [4]. Heterozygous deletion mutant *FKS1/fks1*Δ was constructed by polymerase chain reaction (PCR) product–directed disruption using template plasmid pFA-URA3 and primers FKS1-S1 and FKS1-S2 [5]. Deletion was confirmed by PCR with primer sets (FKS1-G1,U2 and U3,FKS1-G4). Overexpression strain *TDH3-FKS1* was constructed by transforming strain Day185 with PCR products from template pCJN542 and primers FKS1-F-TDH3-OE and FKS1-R-TDH3-OE [6]. Correct construct placement was confirmed with primers FKS1-OE-F-det and Nat-OE-R-det2-CJN. The MY2378A strain (*TET-FKS1*) has been described elsewhere [7]. Strain C48 (*FKS1-S645F*) has homozygous mutation C1934T of *FKS1*, which renders the strain resistant to echinocandins [8].

RNA isolation and real-time PCR methods were completed as described elsewhere [9]. Strains were grown in RPMI-MOPS (Roswell Park Memorial Institute 1640 medium 3-[N-morpholino] propanesulfonic acid) at 37°C, with orbital shaking at 200 revolutions per minute for 6 h. The data analysis was completed using the C_t (2_t^{-C}) method, which generated data as transcript fold-change normalized to a constitutive reference gene transcript (*ACT1*) and relative to the reference strain.

A tetrazolium salt (XTT- 2,3-Bis-(2-methoxy-4-nitro-5-sulfophenyl)-2H-tetrazolium-5-carboxanilide inner salt) reduction assay was used to assess biofilm response to fluconazole (at a concentration of 4–1000 μg/mL) [2]. A microbroth susceptibility method was used to test the impact of matrix on the activity of fluconazole (concentration, 0.125–128 μg/mL) against planktonic *Candida* [2]. Matrix was harvested from 6-well biofilms, concentrated 10-fold by vacuum centrifugation, heat treated at 60°C (10 min), and added to the assay.

A rat central venous catheter infection model was used for in vivo biofilm studies [9,10]. To measure glucan, blood was

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drawn from the catheter at 24 h. For drug treatment experiments, the viable burden was determined after installation of fluconazole (250 μg/mL) into the catheter for 24 h [9]. Scanning and transmission electron microscopy were undertaken as described elsewhere [9].

A murine-disseminated candidiasis model was used as an in vivo nonbiofilm comparison [11]. Mice were injected with 10^5 cells/mL via the tail vein and treated (3 per group) with 1 of 3 fluconazole dosing regimens (3.1, 12.5, or 50 mg/kg/12 h) for 24 h. The total body burden was estimated from the viable burden in mouse kidneys.

Fractionation by alkali-extraction and enzymatic digestion was used to measure cell wall glucan [2]. For extracellular glucan analysis, a Glucatell or Fungitell (1,3)-Beta-D-Glucan Detection Reagent Kit (Associates of Cape Cod, Inc.) was used [2,10].

A ^{3}[H]-labeled fluconazole accumulation protocol was adapted for biofilm use [12]. Biofilms were grown in 6-well plates for 48 h. To degrade the glucan-rich matrix, 24 h biofilms were treated with β-1,3 glucanase (0–6 U/mL) for an additional 24 h [2]. Fluconazole was prepared by adding 30 μCi to 18 mL of RPMI-MOPS (1,686,500 disintegrations per minute per milliliter). After addition of 0.6 mL to each washed biofilm, plates were incubated for 30 min at 37°C with 50 revolutions per minute. Biofilms were washed, dislodged, and collected. After biofilm disruption by vortexing and sonication (10 min), cells were pelleted (4500 g for 20 min) to collect the soluble matrix. Experiments were performed 2–4 times with liquid scintillation counting (TRI-CARB 2100TR; Packard). Data were normalized for dry cell weight.

Results. We examined a collection of mutants with varied *FKS1* expression to discern the role of *FKS1* in biofilm formation. The strains produced similar biofilms in vitro in the wells of polystyrene plates and in vivo on the luminal surfaces of rat venous catheters (data not shown). We constructed a heterozygous deletion mutant, *FKS1/fks1*Δ, with reduced *FKS1* expression (30%, by real-time PCR). However, we were not successful in constructing a *FKS1* null mutant, which is presumed essential in *C. albicans* [7]. We therefore used the *TET-FKS1* mutant with 1 *FKS1* allele under control of a tetracycline repressible promoter and 1 allele deleted to further explore *FKS1* function [7]. The *TET-FKS1* strain formed a biofilm under repressed and nonrepressed conditions. Likewise, overexpression of 1 *FKS1* allele (3.8-fold by real-time PCR) by an inserted *TDH3* promoter did not impact biofilm formation. The final strain examined, *FKS1-S645F*, containing homozygous point mutations in *FKS1* conferring reduced glucan synthase activity, similarly produced biofilms [8]. Each of the strains generated both yeast and hyphae (data not shown).

We next tested the impact of *FKS1* disruption on biofilm drug resistance using an XTT reduction assay. Reference strain biofilms were resistant to fluconazole concentrations (1000 μg/mL) that were >2000 times higher than those to which the planktonic forms were susceptible (Figure 1*A*; data not shown). However, *FKS1/fks1*Δ biofilms were reduced by >80% after 48 h of treatment with fluconazole at a concentration of 250 μg/mL. The *FKS1-S645F* mutant with reduced glucan synthase capacity recapitulated the susceptible biofilm phenotype. For both strains, dose-dependent biofilm reduction was observed with fluconazole concentrations as low as 4 μg/mL (data not shown). Like the reference strain, the *TDH3-FKS1* overexpression strain was maximally resistant to fluconazole. Heterozygous disruption of *FKS1* did not alter planktonic fluconazole susceptibility. Under planktonic conditions, minimum inhibitory concentrations for *FKS1/fks1*Δ and the reference strain were identical (0.25 μg/mL).

We used the *TET-FKS1* strain to further gauge how the expression level of *FKS1* impacted fluconazole resistance. *TET-FKS1* biofilms with doxycycline-repressed *FKS1* were more susceptible to fluconazole than nonrepressed controls (Figure 1*B*). Optimum *FKS1* repression for fluconazole susceptibility occurred at doxycycline concentrations of 7.5–30 ng/mL, with up to 85% biofilm reduction. These doxycycline concentrations did not impact the reference strain.

Using an in vivo vascular catheter biofilm model, we similarly tested the impact of *FKS1* mutation on biofilm resistance. Treatment with fluconazole (250 μg/mL) in the catheter lumen was ineffective against reference strain biofilms but decreased the viable burden in *FKS1/fks1*Δ biofilms by 100-fold (Figure 1*C*). This altered fluconazole susceptibility was specific to biofilm cells. Comparison of the reference strain and the *FKS1/fks1*Δ mutant in a non-biofilm disseminated candidiasis model found no difference in drug efficacy across a wide range of fluconazole doses (3.1–50 mg/kg for 12 h) (Figure 1*D*).

The biochemical impact of *FKS1* modulation on the cell wall and biofilm matrix was evaluated next. Using alkali extraction and enzymatic digestion, we measured biofilm cell wall β-1,3 glucan. Reductions of ~30% were observed for the *FKS1/fks1*Δ and *FKS1-S645F* biofilm cell walls, compared with the reference strain (Figure 2*A*). Doxycycline *FKS1* repression of the *TET-FKS1* strain lowered the amount of cell wall glucan by >50%, compared with the untreated control strains. Cell wall ultrastructure was explored with transmission electron microscopy. Discernible microscopic cell wall changes were not observed among the study strains by this method (data not shown).

Production of extracellular glucan, including matrix glucan, was quantified in the *FKS1* mutant biofilms using a limulus lysate assay. Both supernatant and matrix glucan production were reduced in the *FKS1/fks1*Δ biofilms by ~60% (Figure 2*B*). It is worth noting that the *TDH3-FKS1* biofilms overexpressing *FKS1* produced more than 10-fold more glucan. Using a rat vascular catheter biofilm model, we confirmed a role for *FKS1*

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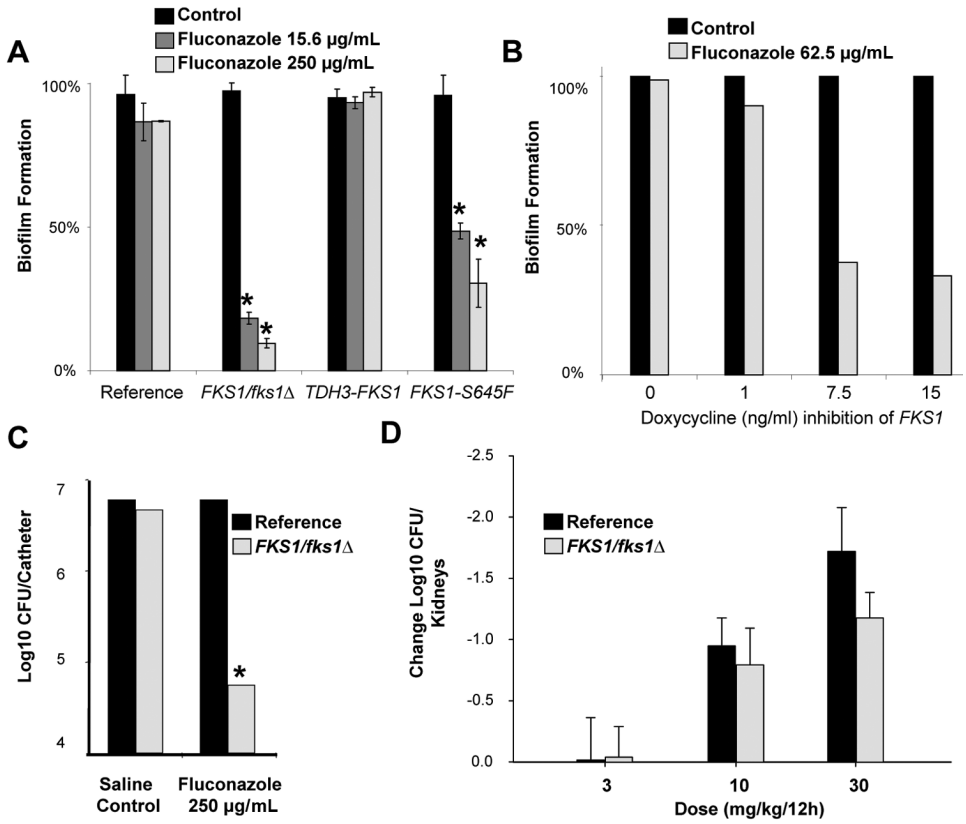

Figure 1. *FKS1* is required for fluconazole biofilm resistance in vitro and in vivo. *A,* The *FKS1/fks1*Δ and *FKS1-S645F* biofilms are more susceptible to 48 h of fluconazole treatment. Assays were performed on 2 occasions in triplicate. *B,* The *TET-FKS1* mutant biofilm is more susceptible to 24 h of fluconazole treatment during repression of *FKS1* by doxycycline at a concentration of 15 ng/mL. Data are from 1 of 3 checkerboard assays that were performed on 3 occasions. *C,* The *FKS1/fks1*Δ biofilm is susceptible to fluconazole in vivo. Biofilms in rat catheters were treated with fluconazole inside the catheter lumen. Data represent the mean value of 2 replicated experiments. *D,* The *FKS1/fks1*Δ mutant and reference strains are equally susceptible to fluconazole in a nonbiofilm disseminated candidiasis model. Four animals were used in each group. Statistical significance was determined by analysis of variance with pairwise comparisons using the Holm-Sidak method; standard deviations are shown. $^*P < .05$.

in extracellular glucan production in vivo. High-magnification scanning electron micrographs of the biofilms growing in rat venous catheters were consistent with these findings. Compared with the reference strain, the *FKS1/fks1*Δ biofilm appeared to have less adherent matrix material, whereas the *TDH3-FKS1* biofilm had more material (Figure 2*C*).

We next tested the hypothesis that biofilm matrix and specifically β-1,3 glucan were responsible for the drug resistance observed during biofilm growth. We used a microbroth susceptibility assay to determine the impact of matrix glucan on planktonic cell resistance [2]. Addition of the reference strain matrix to planktonic cells rendered cells more resistant to fluconazole than the addition of matrix from the strain that produced less matrix β-1,3 glucan (*FKS1/fks1*Δ) (Figure 2*D*). This suggests that the matrix glucan alone is responsible for a degree of resistance observed during biofilm growth and that *FKS1* is necessary for this resistance.

A radio-labeled fluconazole sequestration assay was then designed to measure a biofilm component-antifungal interaction by fluconazole within the intact biofilm and individual biofilm components. Approximately 50% less fluconazole was associated with the intact *FKS1/fks1*Δ and *FKS1-S645F* mutant biofilms than with the reference strain biofilm (Figure 2*E*). Over 70% more fluconazole accumulated in the *TDH3-FKS1* overexpression strain biofilm. Nearly all the radioactivity localized to the matrix, suggesting sequestration of the fluconazole by the extracellular matrix. Unfortunately, radioactivity levels in the intracellular component were below the level of detection.

Using the same assay, we tested the impact of matrix β-1,3 glucan modification on fluconazole sequestration. Reference strain biofilms were treated with β-1,3 glucanase at concentrations known to enhance the activity of fluconazole [2]. Treatment with glucanase significantly reduced the amount of radioactive fluconazole sequestered by the biofilm matrix in a dose-dependent manner, further supporting a glucan-antifungal drug interaction (Figure 2*F*).

Although the current observations strongly suggested that the impact of *FKS1* modulation was due to β-1,3 glucan se-

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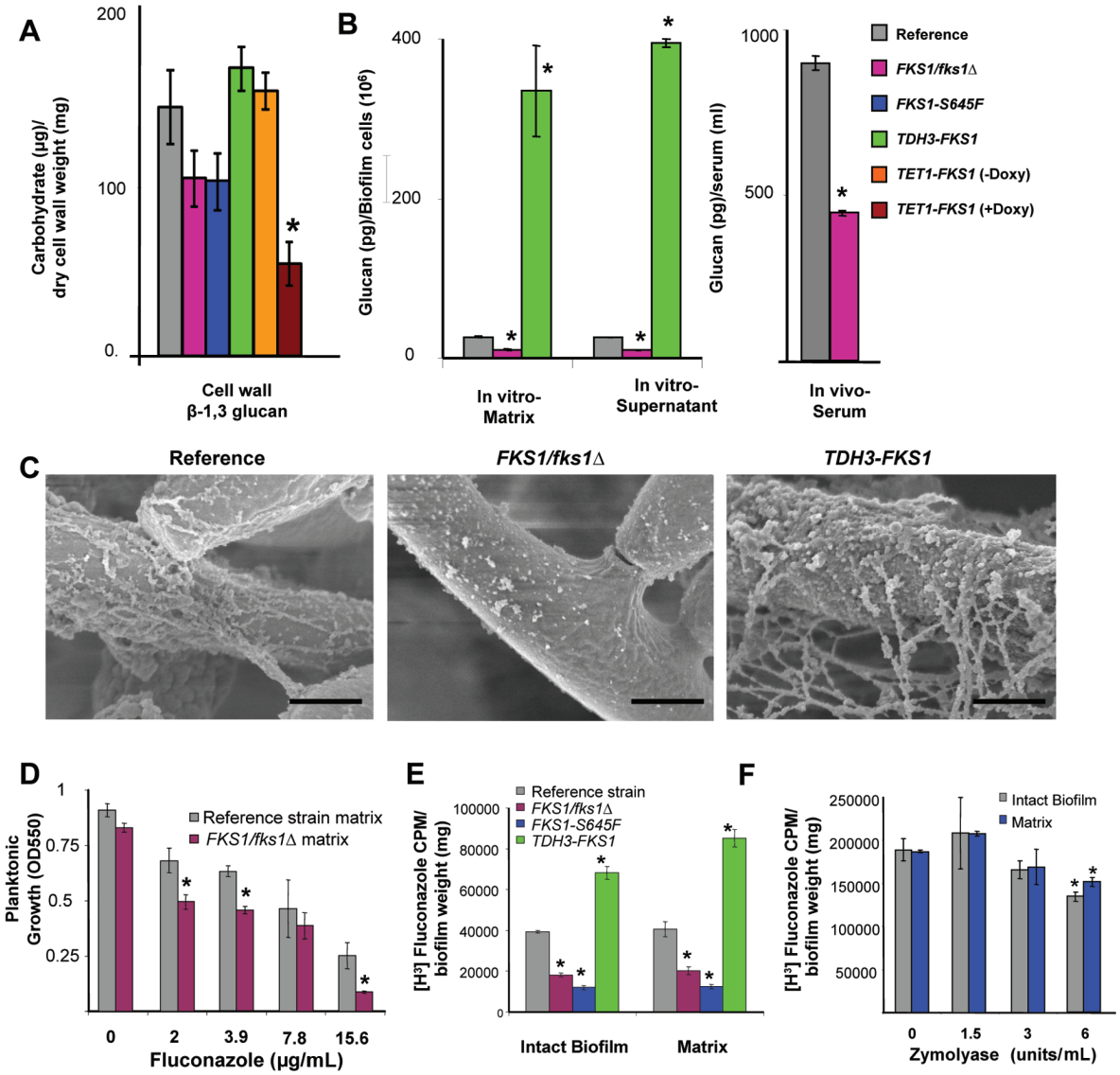

Figure 2. Fks1p produces biofilm matrix glucan which confers resistance to nonbiofilm cells by drug sequestration. *A*, Modulation of *FKS1* impacts biofilm cell wall β-1,3 glucan. Measurements were determined with the phenol sulfuric acid method after alkali-extraction and enzymatic digestion of biofilm cell walls. Assays were performed in triplicate. *B*, *FKS1* is required for extracellular β-1,3 glucan production in biofilms. Supernatant and matrix were collected from in vitro biofilms and β-1,3 glucan was measured using a limulus lysate based assay. A rat venous catheter model was used for in vivo experiments and serum was collected for glucan determination. Glucan assays were preformed in triplicate. *C*, High-magnification scanning electron microscopy ($\times$15,000) of rat catheter biofilms demonstrate the impact of *FKS1* modulation on matrix production. Scale bars represent 1 μm. *D*, Planktonic cells treated with reference strain matrix are significantly more resistant to fluconazole than those treated with *FKS1/fks1*Δ biofilm matrix. Matrix was harvested from *FKS1/fks1*Δ and reference strain biofilms and added to nonbiofilm, planktonic cells in the presence of escalating fluconazole concentrations. Matrix was normalized for each biofilm. Assays were preformed on 2 occasions in triplicate. *E*, *FKS1* is required for matrix sequestration of [H^3] fluconazole. Intact biofilms grown from the glucan modified strains were exposed to [H^3] fluconazole, washed, and harvested. Scintillation counting was used to determine the fluconazole concentration in the intact biofilms and the isolated matrix. Assays were performed on 2 occasions in triplicate. *F*, Degradation of matrix β-1,3 glucan disrupts sequestration of [H^3] fluconazole. Biofilms were treated with escalating concentrations of glucanase prior to [H^3] fluconazole exposure. Statistical significance was determined by analysis of variance with pair-wise comparisons using the Holm-Sidak method; standard deviations are shown. $^*P < .05$.

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questration of antifungals, we also considered the possibility that *FKS1* disruption may lead to a breach of cell wall integrity, rendering cells more susceptible to stress-inducing agents, such as antifungal drugs. We measured the planktonic and biofilm susceptibility of the reference strain and the *FKS1/fks1*Δ mutant to a variety of cell stressors, including hydrogen peroxide, congo red, ethanol, sodium dodecyl sulfate, and hyperosmotic stress. Differences in susceptibility were not detected between the 2 stains (data not shown).

Discussion. *Candida* biofilm cells are embedded in a protective extracellular matrix and exhibit resistance to commonly used anti-infective agents [13–15]. The mechanism for this resistance in *Candida* biofilms appears to be multifactorial [13–15]. However, the contributions of previously identified mechanisms are quantitatively modest, and a large gap remains in our understanding of *Candida* biofilms.

Our findings show that glucan synthesis by Fks1p is critical for biofilm-specific drug resistance in *C. albicans*. As in planktonic cells, in biofilms glucan synthase Fks1p is responsible for production of cell wall β-1,3 glucan during biofilm growth. However, we found this protein to have a unique function in *Candida* biofilms. It also manufactures glucan, which is incorporated into an adhesive, carbohydrate-rich matrix. By producing matrix β-glucan capable of sequestering antifungals, a drug "sponge" prevents the agent from reaching its target, allowing biofilm cells to survive astonishingly high drug exposures. In addition, this matrix glucan has shown to be a potential diagnostic marker for *Candida* biofilm infection in an animal model [10].

Previous investigations have postulated and explored the contribution of the biofilm matrix to antifungal diffusion through *Candida* biofilms [13]. Pivotal studies by the Douglas group [13] identified a relationship between drug susceptibility and the matrix quantity visualized by microscopy. Additional studies have found some slowing of antifungal transit using a filter disk assay. However, previous studies had not addressed the biochemical or genetic basis for this phenomenon.

Defining the chemical composition and genetic regulation of *Candida* biofilm matrix is a promising step for development of diagnostic testing and treatment for devastating device-associated infections. Our findings suggest azoles and echinocandins, which target Fks1p, may be synergistic in biofilms. We envision future studies to address the role of this mechanism for other antifungal drug classes and determine the precise chemical nature of the drug-matrix interaction. Agents designed to disrupt this interaction should prevent or eradicate recalcitrant device-associated infections. In addition, information regarding the matrix composition may be used to design diagnostic tests specific to *Candida* biofilm-associated infections.

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