Interface of *Candida albicans* Biofilm Matrix-Associated Drug Resistance and Cell Wall Integrity Regulation

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Received 24 May 2011/Accepted 1 June 2011

*Candida albicans* frequently infects medical devices by growing as a biofilm, i.e., a community of adherent organisms entrenched in an extracellular matrix. During biofilm growth, *Candida* spp. acquire the ability to resist high concentrations of antifungal drugs. One recently recognized biofilm resistance mechanism involves drug sequestration by matrix β-1,3 glucan. Using a candidate gene approach, we investigated potential *C. albicans* β-1,3-glucan regulators, based on their homology to *Saccharomyces cerevisiae*, including SM1 and protein kinase C (PKC) pathway components. We identified a role for the SM1 in the biofilm matrix glucan production and development of the associated drug resistance phenotype. This pathway appears to act through transcription factor Rlm1p and glucan synthase Fks1p. The phenotypes of these mutant biofilms mimicked those of the smi1Δ/smi1Δ biofilm, and overexpression of *FKS1* in the smi1Δ/smi1Δ mutant restored the biofilm resistant phenotype. However, control of this pathway is distinct from that of the upstream PKC pathway because the pck1Δ/pck1Δ, bck1Δ/bck1Δ, mkk2Δ/mkk2Δ, and mke1Δ/mke1Δ biosilms retained the resistant phenotype of the parent strain. In addition, resistance to cell-perturbing agents and gene expression data do not support a significant role for the cell wall integrity pathway during the biofilm formation. Here we show that Sm1p functions in conjunction with Rlm1p and Fks1p to produce drug-sequestering biofilm β-glucan.

Our work provides new insight into how the *C. albicans* biofilm matrix production and drug resistance pathways intersect with the planktonic cell wall integrity pathway. This novel connection helps explain how pathogens in a multicellular biofilm community are protected from anti-infective therapy.

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*Candida* spp. are an increasing common cause of bloodstream infection in hospitalized patients (42). The capacity to grow in a biofilm state allows these pathogenic fungi to adhere and thrive on medical devices, such as venous catheters, urinary catheters, and dentures (12, 19). The communities of adherent cells become encased in an extracellular matrix, and the cells within the biofilm show extreme resistance to antifungal drugs and host defenses. The biofilm lifestyle allows *Candida* spp. to cause device-associated infections in otherwise healthy hosts (21).

Compared to planktonic, nonbiofilm cells, *Candida albicans* biofilm cells are up to 1,000-fold more resistant to antifungals (7, 22, 28, 44). The treatment-recalcitrant phenotype associated with the *Candida* biofilm state is responsible in part for the high morbidity and mortality observed for patients hospitalized with these infections. Biofilm resistance studies have shown the relevance of several mechanisms important in planktonic resistance in accounting for a portion of this biofilm phenotype (6, 28, 43). It is clear from these studies that *C. albicans* biofilm resistance involves contributions from a combination of mechanisms (1, 2, 8, 22, 28, 43, 47, 50). Studies exploring biofilm resistance have also investigated and identified a link between assembly of an extracellular matrix and biofilm resistance (1, 2, 8, 34, 47, 51). Our recent findings show that glucan synthesis is critical for biofilm-specific drug resistance in *C. albicans* (32, 34). Both production of β-glucan in the biofilm matrix and antifungal resistance to each of the four commercially available antifungal drug classes require β-1,3-glucan synthase gene *FKS1/GSC1*. Disruption of this process decreases drug sequestration in the matrix, rendering biofilms susceptible to antifungal agents. In the proposed resistance model, the matrix glucan covering the biofilm cells is capable of sequestering antifungal drugs and prevents them from reaching their targets. This mechanism was found to account for a large percentage of the drug resistance phenotype during biofilm growth.

Our goal is to identify genes and pathways that control biofilm matrix production and drug resistance in *C. albicans*. Because cell wall pathways are relatively conserved among fungi, we chose to investigate genes known to function in *Saccharomyces cerevisiae* glucan production (15). The protein kinase C (PKC) pathway in yeast has been shown to be important for downstream control of Fks1p and cell wall glucan production in response to stress (26). In addition, this gene network is important for cell wall integrity (26). The function of this pathway in yeast led us to ask two questions. First, is the yeast pathway conserved in *C. albicans* biofilms? Second, does alteration in cell wall integrity during *C. albicans* biofilm growth contribute to drug resistance?

Here we show that the downstream components of the yeast PKC pathway, specifically, SM11, RLML1, and FKS1, are important for manufacture of *C. albicans* cell wall and matrix β-1,3-glucan during biofilm growth. SM11 appears to regulate glucan production through expression of *FKS1* glucan synthase. Without production of biofilm β-1,3-glucan, biofilm cells exhibit...
increased susceptibility to antifungal drugs. Our findings suggest that the matrix production pathway intersects with cell wall integrity regulation but that this regulation is distinct from that of the PKC pathway. Because matrix production is integral for biofilm formation and drug resistance, development of drugs targeting this pathway is a potential strategy for generating antifungals effective against biofilm fungal infections.

MATERIALS AND METHODS

Media. Strains were stored in 15% (vol/vol) glycerol stock at −80°C and maintained on yeast extract-peptone-dextrose (YPD) medium with uridine (1% yeast extract, 2% peptone, 2% dextrose, and 80 µg/ml uridine) prior to experiments. C. albicans transformants were selected on synthetic medium (2% dextrose, 6.7% yeast nitrogen base [YNB] with ammonium sulfate, and auxotrophic supplements) or on YPD plus clonNat (2% Bacto peptone, 2% dextrose, 1% yeast extract, and 400 µg/ml clonNat [Werner Bioagents]) (37). Prior to biofilm experiments C. albicans strains were grown at 30°C in YPD and biofilms were grown in RPMI 1640 buffered with morpholinepropanesulfonic acid (MOPS) (31).

Strains and strain construction. The C. albicans strains used in these studies are listed in Table 1. Strains SF004a (bck1Δ/hisG), VIC1175 (mkk1Δ/hisG), VIC1167 (mkk1Δ/hisG), VIC1156 (mkk1Δ/hisG), BRY429 (dm1Δ/hisG), and VIC1075 (cas5Δ/hisG) were generously provided by A. Mitchell (9, 10). Their construction has been previously described (10, 36). Heterozygous mutant strain JEN118 (SM11/SM1Δ) and the smi1Δ/SM1Δ homozygous mutant strain were constructed from the parent SN152 using disruption marker cassettes and fusion PCR, as previously described (39). For the first round of PCR, primers SM1 F1 (CATTCTTCGTTAATATTG3T) and SM1 F4 (GTCAGGGCCCG CATCCTCTGCTCATCTCAACGAACATGACA) were paired with SM11 F3 (CAGCGGCAGGCTAGCGCTAGGAGAAAGAAGAAGAAGAGAAC) and SM11 F6 (CTCATCAACAGAAAAGATTG) respectively, to amplify the homologous sequences flanking SM11 from template genomic DNA. Universal primers 2 (CCGCTCTAGAGCCGAGCTACCTGGTATGGTGATGATGCTG) and 5 (CAGAGGAAGCCGCGCTGACAGCTCGAGATGATGCTG) were used to amplify Candida dubliniensis HIS1 or Candida maltosa LEU2 with template pSN52 or pSN40, respectively. Flanking sequences were purified with the QiAquick PCR purification kit (Qiagen), and the markers were purified with the AsyPrep DNA gel extraction kit (Aygen Biosciences). Fusion reaction mixtures were assembled using purified templates, SM11 F1 and F6 primers, and Estu polymerase (Takara). Following lithium acetate transformation, correct insertion was confirmed with SM11 Fusion US check (TATATATTCAAGCGCAAAGAA) and SM11 Fusion DS check (TGAACACCGATACATAATG) paired with HIS1 and LEU2 internal primers, as described previously (39).

A cassette for the smi1Δ/SM1Δ+pSM11 complementation strain was constructed by fusion PCR using plasmid pSN105. For the first round of PCR, primers C1 (GCCGAAAGTGCAGCTACTGTCATG) and C4 (GTCAGGGCCCGCGCGGCTAGCCTGAGGAGAAAGAAGAAGAAGACG) were paired with C3 (CGAGGGCGCTGTACAGCTAGCAGGAGGAGGAGGAGGAGGAGG) and C6 (GAGGAGCGTACCTGAGGAGA) respectively, to amplify the homologous sequences flanking C. albicans LEU2 and the C. dubliniensis ARG4 marker using template pSN105. Primers SM11 C2 (CGCTCTCTGCTAGCGCTAGGAGAAAGAAGAAGAAGAGAAC) and SM11 C5 (CGAGGGCGCTGTACAGCCTGAGGAGGAGGAGGAGGAGGAGG) were used to amplify SM11 from genomic
cycles of 94°C for 15 s and 60°C for 1 min. Reactions were performed in the iQ5 PCR detection system (Bio-Rad) with the following primer and probe sets designed using Primer Express (Applied Biosystems, Foster City, CA) for the RNEasy Minikit (Qiagen) and quantitated using a NanoDrop spectrophotometer. TaqMan primer and probe sets designed using Primer Express (Applied Biosystems, Foster City, CA) were used in the RNeasy Minikit (Qiagen) to yield a template DNA. Products were gel purified, and fusion reaction mixtures were assembled using purified templates, C1 and C6 primers, and Extaq polymerase (Takara). Following transformation into the sml1∆/sml1∆ mutant, correct insertion of the cassette containing C. albicans ARG4 and the C. albicans SM1, flanked by sequences homologous to C. albicans LEU2, was confirmed with check primers SM1 end Forward (TGAACCCTGGAATAAGTGGATT) and CaLeU2downdkl det R (CGAGGCGCACATTACATCTCACCAG). The sml1∆/sml1∆-TDH3-FKS1 strain (JEN172) was constructed using plasmids and primers as previously described (34, 37, 38). Briefly, a cassette containing a TDH3 promoter was inserted upstream of one FKS1 allele in the sml1∆/sml1∆ mutant.

RNA isolation and real-time RT-PCR expression analysis. RNA was collected from biofilm cells grown in 6-well plates, as described below. RNA was purified using the RNasy Mini kit (Qiagen) and quantitated using a NanoDrop spectrophotometer. TaqMan primer and probe sets designed using Primer Express (Applied Biosystems, Foster City, CA) for ACT1, ACT1 RT For (AGCTTTGTTCAGACCAGCTGATT), and SMI1 probe (5'-ACACCATGGGCAACACCAGCA-3';) were used in an iQ5 PCR detection system (Bio-Rad) with the following program: 50°C for 30 min, initial denaturation at 95°C for 15 min, and then 40 cycles of 94°C for 15 s and 60°C for 1 min. Reactions were performed in triplicate. The expression of each gene relative to that of ACT1 is presented. The quantitative data analysis was completed using the 2^-ΔΔCT method (23). The comparative expression method generated data as transcript fold change normalized to a constitutive reference gene transcript (ACT1) and relative to the reference strain.

In vitro biofilm model. Biofilms were grown in 6-well or 96-well flat-bottom polystyrene plates as previously described (31, 33). The C. albicans inoculum (10^5 cells/ml) was prepared by growth in YPD with uridine overnight at 30°C, followed by dilution in RPMI-MOPS based on hemocytometer counts. For 6-well plates, 1 ml of culture was inoculated in each well. After a 60-min adherence period at 30°C, the nonadherent inoculum was removed and 1 ml of fresh medium (RPMI-MOPS) was applied to each well. Plates were incubated at 37°C for 48 h on an orbital shaker set at 50 rpm. Medium was removed and fresh medium was added midway through the incubation period.

In vivo C. albicans venous catheter biofilm model. A jugular vein rat central venous catheter infection model was used for in vivo biofilm studies (5, 30). Candida strains were grown to late logarithmic phase in YPD at 30°C with orbital shaking at 200 rpm for 24 h followed by a 24-h recovery period after catheter implantation. The catheter infection model was used for scanning electron microscopy (SEM) studies, the catheter segments were mounted, gold coated, and imaged in a scanning electron microscope (JEOL JSM-6100) at 10 kV. The images were assembled using Adobe Photoshop 7.0.1.

In vitro disseminated C. albicans infection model. A neutropenic murine disseminated candidiasis model mimicking systemic Candida infection was used to assess strain virulence and drug susceptibility in the nonbiofilm setting (3). Mice were injected with 10^6 CFU/ml C. albicans via the tail vein. Animals were treated with one of three fluconazole subcutaneous regimens (3.1, 12.5, or 50 mg/kg/12 h) for 24 h; the regimens were chosen based upon treatment efficacy from previous studies (4). Total body Candida burden was estimated by measuring viable burden in kidney homogenates as previously described (3, 4).

In vitro biofilm and planktonic antifungal susceptibility testing. A tetrazolium salt (XTT [2,3-bis-(2-methoxy-4-nitro-5-sulfophenyl)-2H-tetrazolium-5-carboxanilide inner salt]) reduction assay was used to measure in vitro biofilm drug susceptibility (31, 33, 45). Biofilms were formed in the wells of 96-well microtiter plates, as described above. After a 6-h biofilm formation period, the biofilms were washed with phosphate-buffered saline (PBS) twice to remove nonadherent cells. Fresh RPMI-MOPS and drug dilutions were added, followed by additional periods of incubation (48 h). The antifungal contents included fluconazole at 4 to 1,000 μg/ml, amphotericin B at 0.008 to 2 μg/ml, and anidulafungin at 0.002 to 8 μg/ml. Drug treatments were repleted after 24 h, and plates were incubated for an additional 24 h (34). Following treatment with 90 μl XTT (0.75 mg/ml) and 10 μl phenazine methosulfate (320 μg/ml) for 1 h, absorbance at 492 nm was measured using an automated plate reader. The percent reduction in biofilm growth was calculated using the reduction in absorbance compared to that of controls with no antifungal treatment. Assays were performed in triplicate, and significant differences were measured by analysis of variance (ANOVA) with pairwise comparisons using the Holm-Sidak method.

The CLSI M27 A3 broth microdilution susceptibility method was used to examine the activities of antifungal agents and biocides against planktonic C. albicans (29). The concentration ranges studied were as follows: fluconazole, 0.125 to 128 μg/ml; anidulafungin, 0.03 to 32 μg/ml; fluconazole, 0.03 to 32 μg/ml; and amphotericin B, 0.03 to 32 μg/ml. Endpoints were assessed after 24 h by visible growth.

Planktonic and biofilm biocide susceptibility testing. The CLSI M27 A3 broth microdilution susceptibility method was used to examine the activities of agents associated with cell wall stress against planktonic C. albicans (29, 33). Agents with various mechanisms of action known to impact cell integrity were included (48). MICs were recorded visually and by absorbance reading at 550 nm. A 96-well XTT assay, as described above, was used for measurement of the biocidal response to stress-inducing agents. Concentrations acquired for a 50% reduction in XTT absorbance (50% effective concentration [EC50]) was recorded as the endpoint. Assays were performed in triplicate. The following concentration ranges were tested for planktonic and biofilm studies: calcofluor white, 0.2 to 200 μg/ml; ethanol, 0.01 to 50%; and sodium dodecyl sulfate (SDS), 0.001 to 2%.
(1,3)-beta-D-glucan detection reagent kit (Associates of Cape Cod, MA) per the manufacturer’s directions.

Accumulation of [H3]fluconazole into C. albicans biofilms. A radiolabeled-fluconazole accumulation protocol was adapted for biofilm use as previously described (25, 34, 49). Biofilms were grown in 6-well plates, as described above, for 48 h. The biofilms were washed with sterile water twice. For stock solution preparation, radioactive [H3]fluconazole (Moravek Biochemicals, 50 μM, 0.001 mC/ml in ethanol) was diluted 100-fold in water. The stock solution was then diluted 6-fold in RPMI-MOPS, and each biofilm well received a total of 600 μCi/ml in ethanol) was diluted 100-fold in water. The stock solution was then diluted 100-fold in water. The stock solution was then diluted 6-fold in RPMI-MOPS, and each biofilm well received a total of 600 μC/ml of medium to yield a total of 8.48 × 106 cpm of [H3]fluconazole. After incubation for 30 min at 37°C and orbital shaking at 50 rpm, unlabeled (cold) 20 μM fluconazole in RPMI-MOPS was added and biofilms were incubated for an additional 15 min. Biofilms were then washed twice with sterile water, gently dislodged with a sterile spatula, and collected as intact biofilms for scintillation counting. The biofilms were then disrupted by vortexing and sonication for 10 min to separate cells and matrix. Following centrifugation at 4,500 × g for 20 min, cells were separated from the soluble matrix material. Cells were disrupted by bead beating, and the intracellular and cell wall portions were collected by centrifugation. The fractions were then suspended in ScintiSafe 30% LSC cocktail (Fisher Scientific) and counted in a Tri-Carb 2100TR liquid scintillation analyzer (Packard). Student’s t test was used to determine statistical significance of differences between strains.

| Mutation | Description | Mutant phenotypea | Biofilm formationb | Matrix glucan productionc | Biofilm fluconazole resistanced |
|----------|-------------|-------------------|--------------------|--------------------------|-----------------------------|
| None (reference strain) | Wild type | ++ | ++ | ++ |
| C. albicans homologs of S. cerevisiae components of the PKC pathway | | | | |
| pkc1Δ/pkc1Δ | Mammalian PKC homolog | ++ | ++ | ++ |
| mkk2Δ/mkk2Δ | MAPKKKd | ++ | + | ++ |
| bck1Δ/bck1Δ | MAPK | + | + | + |
| mkk1Δ/mkk1Δ | Regulator of glucan synthesis | + | – | – |
| smi1Δ/smi1Δ | Transcription factor | + | – | – |
| rm1Δ/rm1Δ | β-1,3-Glucan synthase | + | – | – |
| FKS1/fok1Δ | Transcription regulator | + | + | + |

Cell wall regulator unique to C. albicans cas5Δ/cas5Δ | | |

* + +, value is within 10% of reference strain level; +, value is 10 to 30% below reference strain level; –, value is less than 30% of reference strain level.

b Measured using XTT reduction assay in a 96-well plate.

c Measured by glucan limulus lysate assay.

d MAPKKK, mitogen-activated protein kinase kinase.

RESULTS

Role of S. cerevisiae PKC pathway homologs in C. albicans biofilm formation and biofilm-associated drug resistance. We utilized a candidate gene approach to identify regulators of C. albicans β-glucan production, with the goal of determining the control of this process during the development of biofilm drug resistance. Yeast PKC pathway homologs in C. albicans, including PKC1, BCK1, MKK2, MKC1, and SMI1, were chosen based upon demonstrated importance for control of β-1,3-glucan synthesis in S. cerevisiae. We were surprised that disruption of PKC1, BCK1, MKK2, or MKC1 did not affect biofilm drug resistance (Table 2). Each null mutant formed a biofilm in vitro that demonstrated the characteristic drug-resistant phenotype. However, we did identify C. albicans SMI1 as a regulator of biofilm-associated drug resistance. Homozygous deletion of SMI1 produced a biofilm with enhanced susceptibility to antifungal drugs (Fig. 1). While the reference strain biofilm was resistant to the highest fluconazole concentration tested (1,000 μg/ml), the homozygous smi1Δ/smi1Δ biofilm was reduced by 50% upon fluconazole treatment at concentrations of 125 and 250 μg/ml. Differences in susceptibility were observed at concentrations of as low as 7 μg/ml (data not shown). Compared to the reference strain, the smi1Δ/smi1Δ biofilm exhibited increased susceptibility to antifungals from additional drug classes (anidulafungin and amphotericin B) as well (Fig. 2). Complementation of SMI1 partially restored resistance to fluconazole. Disruption of SMI1 did not affect planktonic susceptibility to any of the antifungal agents tested using standard MIC testing methods, suggesting a biofilm-specific role for the gene products in drug resistance (Table 3). Because the phenotype of the smi1Δ/smi1Δ biofilm was distinct from that of the PKC pathway mutants, we also consid-
We further determined the impact of SMI1 on biofilm cell wall β-1,3-glucan production by measuring and comparing the carbohydrate contents of individual biofilm cell wall fractions. Compared to that of the reference strain, the smiΔΔ/smiΔΔ biofilm cell wall was composed of significantly less total glucan (Fig. 3B). Most of the observed difference was accounted for by the 40% decrease in cell wall β-1,3-glucan for the smiΔ/smiΔ biofilm. In addition, a smaller amount of β-1,6-glucan was detected in this mutant cell wall. Reduced biofilm cell wall glucan production was also observed for the SMI1Δ/smiΔΔ mutant. As expected for a heterozygous disruption, the extent of these differences was less than those measured for the homozygous disruption. The extent of the biochemical reduction in cell wall β-1,3-glucan for the smiΔ/smiΔ mutant is similar to the cell wall percent reduction measured by TEM (Fig. 4A). A decrease in cell wall β-1,3-glucan is consistent with the microscopy images demonstrating a thinner electron-lucent cell wall layer.

Impact of SMI1 disruption on biofilm formation and azole susceptibility in an in vivo rat venous catheter. We utilized a rat biofilm catheter model to examine the impact of SMI1 disruption on biofilm formation and matrix production in vivo (5). We included the in vivo model because reports have demonstrated variability in genes needed for biofilm development.

FIG. 3. SMI1 affects biofilm cell wall and matrix glucan content. (A) SMI1 is required for matrix β-1,3-glucan production in biofilms. Matrix samples were collected from in vitro biofilms growing in 6-well polystyrene plates. β-1,3-glucan was measured using a Limulus lysate-based assay. Assays were performed in duplicate on two occasions. (B) Cell walls from reference strain, SMI1Δ/smiΔΔ, and smiΔ/smiΔ mutant biofilms were isolated and fractionated by alkali treatment and enzymatic digestion. ANOVA with pairwise comparisons using the Holm-Sidak method was used to compare carbohydrate of each fraction among the strains. *, P < 0.05. Assays were performed in triplicate on two occasions. Standard deviations are shown.

### TABLE 3. Impact of SMI1 modulation on planktonic drug susceptibility

| Strain                  | MIC (μg/ml) | Fluconazole | Amphotericin B | Fluycytosine | Anidulafungin |
|-------------------------|-------------|-------------|----------------|--------------|---------------|
| Reference               |             | 0.03        | 0.13           | 0.015        |
| smiΔ/smiΔΔ mutant       | 0.5         | 0.06        | 0.06           | 0.06         |
| SMI1Δ/smiΔΔ mutant      | 0.5         | 0.06        | 0.25           | 0.015        |
| smiΔ/smiΔΔ psMI1 mutant | 1           | 0.06        | 0.13           | 0.015        |

* MICs were determined using the CLSI method and endpoint.
under in vitro and in vivo conditions (35). Following a 6-h adherence period and a 24-h growth period, catheter segments were processed for imaging by scanning electron microscopy (SEM). Both the reference strain and the smi1Δ/smi1Δ mutant strain were capable of adhering to the luminal catheter surface and forming heterogeneous biofilms across the surface with comparable total biofilm masses (Fig. 5). Imaging at higher magnification revealed an extracellular matrix material coating the cells, as previously described (34). However, the smi1Δ/smi1Δ biofilm appeared to produce less matrix material than the reference strain. These in vivo images correlate with the smaller amount of biofilm matrix glucan measured for the smi1Δ/smi1Δ in vitro biofilms and support a role for SMII in matrix glucan production and deposition.

In treatment studies, the biofilm susceptible phenotype that we identified for the smi1Δ/smi1Δ mutant in vitro was corroborated in vivo. Following intraluminal fluconazole treatment (512 μg/ml), the Candida viable burden was more than 3-fold lower for the smi1Δ/smi1Δ mutant strain \((2.5 \times 10^5 \text{ CFU/catheter})\) than for the reference strain \((8.5 \times 10^5 \text{ CFU/catheter})\).

**Impact of SMII expression on uptake of \([H^3]\)fluconazole in C. albicans biofilms.** A radiolabeled-fluconazole accumulation assay was used to track fluconazole within the in vitro reference strain and mutant biofilms. Following treatment with [H³]fluconazole, less fluconazole was associated with the intact smi1Δ/smi1Δ mutant biofilm than with the reference strain biofilm (Fig. 6). Nearly all of the biofilm-associated radioactive fluconazole was localized to the biofilm matrix. Unfortunately, radioactivity levels in the intracellular component were below the level of detection (data not shown). The accumulation of drug in the biofilm matrix is consistent with sequestration of the fluconazole by the extracellular matrix. Altered sequestration in the smi1Δ/smi1Δ mutant biofilm suggests a role for this gene product in the drug sequestration process, as has been described for glucan synthase gene FKS1 (34).

**Relationship between FKS1 and SMII.** We considered a link between SMII and Fks1p, a glucan synthase required for C. albicans biofilm glucan production and drug resistance during biofilm growth (34). We examined the collection of mutants with varied SMII expression and measured transcript abundances of both SMII and FKS1 during biofilm growth by real-time RT-PCR. Heterozygous disruption of SMII decreased the SMII transcript abundance to approximately 50% of that for the reference strain (Fig. 7A). Homozygous disruption eliminated transcription entirely, while complementation partially restored SMII transcription. Transcript abundance of FKS1 was decreased by nearly 50% in the smi1Δ/smi1Δ mutant biofilm and by approximately 10% in the SMII/smi1Δ mutant biofilm, consistent with a gene dose effect. To corroborate the connection between SMII and FKS1, we constructed a smi1Δ/smi1Δ mutant biofilm with FKS1 under the control of a TDH3 promoter and hypothesized that overexpression of FKS1 would rescue the biofilm drug susceptibility phenotype. As hypothesized, the smi1Δ/smi1Δ TDH3-FKS1 biofilm was less susceptible to fluconazole treatment than the smi1Δ/smi1Δ parent strain (Fig. 7B). These results support the hypothesis that SMII functions upstream of FKS1 for biofilm glucan production and drug resistance.

A potential relationship between C. albicans Smi1p and Fks1p is predicted based on S. cerevisiae Smi1p/Knr4p, which modulates FKS1 expression via transcription factor Rlm1p (26). To investigate this relationship in C. albicans biofilms, we measured glucan production and drug resistance in the rlm1Δ/rlm1Δ mutant biofilm. Similar to the case for the smi1Δ/smi1Δ and FKS1/fks1Δ mutant biofilms, the rlm1Δ/rlm1Δ mutant exhibited increased susceptibility to fluconazole (Table 2). This increased susceptibility corresponded with decreased matrix glucan, consistent with disruption of drug sequestration by the matrix. Together, the data suggest that Smi1p regulates Fks1p, glucan production, and drug resistance during biofilm growth, likely through Rlm1p.

**Cell wall integrity and SMII.** We considered the possibility that disruption of SMII and cell wall glucan may result in global changes, rendering C. albicans more susceptible to a variety of stressors. We examined biofilm and planktonic organism susceptibility to known cell-perturbing agents (calcofluor white, ethanol, and SDS) (Table 4). With the exception of calcofluor white, the smi1Δ/smi1Δ and reference strains were similarly susceptible during biofilm and planktonic growth. As a complementary method, we examined the transcript levels of four cell wall damage response genes in reference strain and smi1Δ/smi1Δ mutant biofilms (Fig. 8) (40). In the absence of an exogenous stressor, disruption of SMII resulted in a minor change in each of these cell wall damage response genes that
we examined. The highest expression change was approximately 1.5-fold.

**Impact of SMII disruption on growth rate and virulence.**

We measured the impact of SMII disruption on growth rate, filamentation, and virulence, characteristics previously demonstrated to be important for biofilm formation, in a murine disseminated candidiasis model. The smi1Δ/Δ mutant and reference strain grew at similar rates under planktonic conditions (Fig. 9A). Both strains generated hyphae in response to hypha-inducing conditions, including growth in RPMI-MOPS at 37°C and on Spider medium (data not shown). Each of the SMII-modulated strains formed biofilm *in vitro* in the wells of polystyrene plates, and the biofilm quantities were similar at 6 and 24 h based on XTT reduction assay (data not shown). To address the impact of SMII on *C. albicans* virulence, we used a disseminated murine neutropenic candidiasis model (3). Over the 24-h period, the viable burdens of the smi1Δ/Δ mutant and reference strain increased at similar rates and to equivalent magnitudes over the study period (Fig. 9B). To-

FIG. 5. SMII is not required for biofilm formation *in vivo*. The luminal surfaces of rat venous catheters were inoculated with the smi1Δ/Δ mutant or the reference strain. Catheter segments were processed and imaged using SEM. At low magnification (×50), the reference strain and smi1Δ/Δ mutant appear to form similar biofilms extending over the luminal catheter surface. At higher magnification, the smi1Δ/Δ exhibits visually less matrix than the reference strain. Scale bars in the ×50, ×1,000, and ×3,000 images represent 500 μm, 20 μm, and 8 μm, respectively.

FIG. 6. SMII is required for matrix sequestration of [H3]fluconazole. Intact biofilms grown from the glucan-modified strains were exposed to [H3]fluconazole, washed, and harvested. Scintillation counting was performed in triplicate to determine the fluconazole concentrations in the intact biofilms and the isolated matrix. Standard deviations are shown. Student’s *t* test was used to determine statistical significance of differences between strains. *, *P* < 0.05. Standard deviations are shown.
gether, these experiments show the disruption of **SMI1** does not significantly influence growth, filamentation, or virulence in an *in vivo* nonbiofilm infection model.

**DISCUSSION**

*Candida* spp. infect medical devices by attaching to the surface and proliferating as a biofilm. Cells in this environment are embedded in a protective extracellular matrix and exhibit profound resistance to antifungal drugs (11, 14). Because of the lack of effective antifungal therapy against biofilm infections, the recommended treatment for *Candida* biofilm infections is removal of the infected device (42). Defining the pathways and mechanisms of resistance during the biofilm mode of growth is valuable for the design of innovative drug therapies targeted to treat these recalcitrant infections.

The first *Candida* biofilm resistance studies tested mechanisms of resistance known to be important in planktonic systems (6, 28, 43, 50). Although several of these mechanisms were found to be involved in biofilm drug resistance, much of the biofilm resistant phenotype remained an enigma. Subsequent studies have suggested that biofilm drug resistance is multifactorial, with contributions from biofilm-specific processes as well (6, 18, 20, 28, 34, 43, 47, 50). Models have demonstrated phenotypic variability among the cells in heterogeneous biofilms and have identified subsets of exquisitely resistant cells deep in the biofilm (18, 22). These studies suggest that cells throughout the biofilm may even employ different mechanisms of resistance.

Previous investigations postulating a contribution of the biofilm matrix to drug resistance measured antifungal diffusion through *Candida* biofilms (1, 2, 8, 47). Using variable-flow conditions to alter matrix production, the Douglas group identified a correlation between drug resistance and the extent of matrix production. A correlation between drug resistance and the extent of matrix production was also observed in the current study using the *SMI1* disruption strain.

**TABLE 4. Impact of SMI1 disruption on planktonic cell and biofilm susceptibility to biocides**

| Cell type | Strain        | Conc at which cells susceptible to: |
|-----------|---------------|-------------------------------------|
|           |               | Calcofluor | Ethanol | SDS  |
| Planktonic | Reference     | 50        | 0.8     | 0.01 |
|           | *smi1Δ/Δ* mutant | 3.12 | 0.8     | 0.01 |
| Biofilm   | Reference     | 100       | 12.5    | 0.06 |
|           | *smi1Δ/Δ* mutant | 25   | 12.5    | 0.03 |

* MICs were determined using the CLSI method and endpoint.

* The XTT reduction assay was used to determine the drug concentration associated with a 50% reduction in optical density compared to that in the no-drug control wells (EC50).

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**FIG. 7. Modulation of SMI1 affects FKS1 expression in *C. albicans* biofilms. (A) RNA was isolated from reference and mutant biofilms. Real-time RT-PCR assays were used to measure transcript levels in triplicate. Mean results were normalized by *ACT1* RNA measurements performed simultaneously and compared to the reference strain using the method of Livak and Schmittgen (23). Data are shown as a normalized ratio of transcript in the mutant strain divided by that in the reference strain. (B) FKS1 was placed under the control of an inserted TDH3 promoter for overexpression of FKS1 in the homozygous *smi1Δ/Δ* mutant. Biofilms were treated with serial dilutions of fluconazole for 48 h, supplied as a two 24-h doses, and drug impact was determined using an XTT reduction assay. Data are expressed as percent reduction compared to untreated controls. Standard errors are shown. Student’s t test was used to compare the mutant strains at each drug concentration. *, P < 0.05.

**FIG. 8. Expression of cell wall damage genes in *smi1Δ/Δ* mutant and reference strain biofilms. RNA was isolated from reference and mutant biofilms. Real-time RT-PCR assays were used to measure transcript levels in triplicate. Mean results were normalized by *ACT1* RNA measurements performed simultaneously and compared to the reference strain using the method of Livak and Schmittgen (23). Data are shown as a normalized ratio of transcript in the mutant strain divided by that in the reference strain.
biofilm matrix (2, 8). Further studies with filter disk diffusion assays have also revealed a slowing of antifungal transit through Candida biofilms (1, 47). Our previous investigations linked a biofilm matrix carbohydrate, glucan, to a biofilm-specific drug resistance mechanism in C. albicans (32, 34). By producing matrix β-glucan capable of sequestering an antifungal drug, biofilm cells survive extraordinarily high drug concentrations during biofilm growth.

In the current studies, we use a candidate gene approach to explore a role for the PKC pathway in C. albicans matrix β-1,3-glucan production and biofilm resistance. This pathway has previously been described to be a positive regulator of β-1,3-glucan synthesis in S. cerevisiae, but similar studies have not been performed with C. albicans to our knowledge (17). Here we show that SMI1 and RLM1 are required for production of the characteristic drug-resistant phenotype of the biofilm lifestyle. Disruption of SMI1 and RLM1 affects the manufacture of C. albicans β-1,3-glucan matrix and cell wall glucan during biofilm growth. The importance of these genes in S. cerevisiae β-1,3-glucan cell wall synthesis has been well characterized, but their role in C. albicans β-1,3-glucan production and biofilm resistance is a novel finding (16). Surprisingly, the upstream yeast homologs of the PKC pathway were not found to contribute to the biofilm glucan matrix resistance mechanism. Disruption of each of the four kinases in the pathway did not affect biofilm drug resistance or glucan matrix.

Consistent with previous investigations, the current studies support a role for glucan sequestration of antifungals in C. albicans biofilm drug resistance (34). Using a radioactive-fluconazole assay, we were able to track the drug accumulation and demonstrate biofilm sequestration in the matrix material. The finding that modulation of SMI1 affects the amount of matrix-sequestered drug indicates a role for the gene product in this biofilm resistance mechanism. This mechanism is specific to the biofilm mode of growth because planktonic drug resistance is not affected. The ability of biofilm matrix to act as a drug sponge, occupying the drug and preventing its activity, has been described for both bacterial and fungal biofilms (24, 34). In C. albicans biofilms, this activity is mediated, at least in part, through glucan synthase Fks1p.

These studies show that the action of Smi1p includes β-1,3-glucan matrix synthesis upstream of Fks1p. Transcription of FKS1 is modulated by SMI1 expression, and the β-1,3-glucan changes observed with disruption of SMI1 are similar to those described for FKS1/fks1 mutant biofilm (34). Furthermore, we show that overexpression of FKS1 in the smi1Δ/smi1Δ mutant restores the biofilm drug-resistant phenotype. In S. cerevisiae, a link between SMI1/KNR4 and FKS1 upon activation of the cell wall integrity pathway and transcription factor Rlm1p has been described (26). As predicted from S. cerevisiae, disruption of RLM1 in C. albicans produces a biofilm phenotypically similar to the SMI1 and FKS1 mutant biofilms. Together, the findings suggest that the relationship between these gene products is conserved in C. albicans.

Phenotypic studies and transcriptional profiling examining the cell wall integrity pathway in the smi1Δ/smi1Δ mutant suggest a partial link to biofilm matrix production. Disruption of SMI1 affected expression of several cell wall damage response genes, suggesting altered cell wall integrity for the mutant strain in the absence of exogenous stressors. In addition, the smi1Δ/smi1Δ mutant was significantly more susceptible to cell wall perturbation by calcofluor white. However, significant differences between the strains were not observed upon treatment of planktonic or biofilm cells with other cell stressors. We hypothesize that the differential susceptibility to calcofluor white is related to the increase in cell wall chitin in the smi1Δ/smi1Δ mutant (data not shown). Disruption of SMI1 did not affect growth at 37°C or planktonic susceptibility to additional antifungals, including amphotericin B, flucytosine, and anidulafungin. An intact cell wall integrity pathway is required for echinocandin resistance in both C. albicans and S. cerevisiae (10, 46). Therefore, similar planktonic susceptibilities to anidulafungin for the C. albicans smi1Δ/smi1Δ mutant and the reference strain do not suggest an altered cell wall integrity pathway.

Taken together, the data suggest that matrix glucan production and biofilm resistance are modulated by Smi1p and networked to the cell wall integrity pathway. However, regulation of this pathway is distinct from that of the PKC pathway. Further defining the genetic regulation of this Candida biofilm pathway may provide insight into how the organism transforms to this lifestyle and resists antifungal treatment.

ACKNOWLEDGMENTS

We thank A. Mitchell, S. Noble, and C. Nobile for strains and plasmids.
