Characteristics of *Candida albicans* Biofilms Grown in a Synthetic Urine Medium[▽]

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Urinary tract infections (UTIs) are the most common type of nosocomial infection, and *Candida albicans* is the most frequent organism causing fungal UTIs. Presence of an indwelling urinary catheter represents a significant risk factor for UTIs. Furthermore, these infections are frequently associated with the formation of biofilms on the surface of these catheters. Here, we describe the characterization of *C. albicans* biofilms formed in vitro using synthetic urine (SU) medium and the frequently used RPMI medium and compare the results. Biofilms of *C. albicans* strain SC5314 were formed in 96-well microtiter plates and on silicon elastomer pieces using both SU and RPMI media. Biofilm formation was monitored by microscopy and a colorimetric XTT [2,3-bis(2-methoxy-4-nitro-5-sulfophenyl)-2H-tetrazolium-5-carboxanilide] reduction assay. As in biofilms grown in RPMI medium, time course studies revealed that biofilm formation using SU medium occurred after an initial adherence phase, followed by growth, proliferation, and maturation. However, microscopy techniques revealed that the architectural complexity of biofilms formed in SU medium was lower than that observed for those formed using RPMI medium. In particular, the level of filamentation of cells within the biofilms formed in SU medium was diminished compared to those in the biofilms grown in RPMI medium. This observation was also corroborated by expression profiling of five filamentation-associated genes using quantitative real-time reverse transcriptase PCR. Sessile *C. albicans* cells were resistant to fluconazole and amphotericin B, irrespective of the medium used to form the biofilms. However, caspofungin exhibited potent in vitro activity at therapeutic levels against *C. albicans* biofilms grown in both SU and RPMI media.

Candida albicans is a nosocomial pathogen that has a predilection for the urinary tract, where it can cause infections with a broad spectrum of disease severity. It is thereby known to be the most frequent organism causing fungal urinary tract infections (UTIs) (26). Presence of an indwelling urethral catheter represents a significant risk factor for *C. albicans* UTI as these infections are frequently associated with the formation of biofilms on the surface of these catheters (8, 19). *C. albicans* biofilms are highly organized communities of yeast, hyphae, and pseudohyphae attached to the surface of biomaterials and enclosed in a matrix of polysaccharides and are highly resistant to most major classes of antifungal drugs (21).

C. albicans biofilms have been developed on several different model systems in vitro (4, 14, 24, 30). Several experimental variables have been incorporated into these systems in order to mimic conditions in patients, such as flow to simulate blood flow, coating of substrate surfaces with various host blood and salivary proteins, variation in the nutrient medium employed, and use of common device materials (14, 20). In the present study we grew *C. albicans* biofilms in synthetic urine (SU) medium—an in vitro model mimicking an in vivo biofilm on a urinary catheter. Our goal was to study the morphological and architectural characteristics, as well as the antifungal suscepti-

bility profiles, associated with *C. albicans* biofilms grown in SU medium.

MATERIALS AND METHODS

Strain. The *C. albicans* strain used in the present study was wild-type strain SC5314. Cells were stored at -70°C as glycerol stocks and propagated by streaking a loopful of cells onto plate of yeast-peptone-dextrose (YPD) agar (1% [wt/vol] yeast extract, 2% [wt/vol] peptone, 2% [wt/vol] dextrose agar) and incubating overnight at 37°C . Flasks (150 ml) containing 20 ml of YPD liquid medium were then inoculated with a loopful of cells from YPD agar plates and grown overnight in an orbital shaker (150 to 180 rpm) at 30°C . Under these conditions, *C. albicans* grows as budding yeast.

Biofilm growth conditions. *C. albicans* biofilms were formed in RPMI and SU media. SU medium was composed as previously described (6) and consisted of CaCl_2 (0.65 g/liter), MgCl_2 (0.65 g/liter), NaCl (4.6 g/liter), Na_2SO_4 (2.3 g/liter), $\text{Na}_3\text{-citrate}$ (0.65 g/liter), $\text{Na}_2\text{-oxalate}$ (0.02 g/liter), KH_2PO_4 (2.8 g/liter), KCl (1.6 g/liter), NH_4Cl (1.0 g/liter), urea (25.0 g/liter), creatinine (1.1 g/liter), 5% yeast nitrogen base medium (vol/vol), and 2% (wt/vol) glucose. The pH was adjusted to 5.8, and the mixture was sterilized by filtration. RPMI medium (RPMI 1640 medium supplemented with L-glutamine and buffered with morpholinepropanesulfonic acid; Angus Buffers and Chemicals, Niagara Falls, NY), also containing 2% glucose (wt/vol) and adjusted to a final pH of 7.2, was used as a control for comparisons since *C. albicans* biofilms grown in this medium have been described by many different groups and have been extensively characterized (10, 17, 18).

Two different in vitro models were used to assess biofilm growth and drug susceptibility. The first was a microtiter plate-based model that utilizes polystyrene, flat bottomed, 96-microtiter plates (Corning Inc.) as a substrate, as previously described (18). Briefly, cells were grown in YPD medium overnight at 30°C , washed twice with sterile phosphate-buffered saline (PBS; 10 mM phosphate buffer, 2.7 mM potassium chloride, 137 mM sodium chloride, pH 7.4 [Sigma, St. Louis, MO]), and resuspended in RPMI or SU medium at a concentration of 10^6 cells/ml. This inoculum (100 μl) was added to each well of a 96-well flat-bottomed plate. After a 24-h incubation (without shaking) at 37°C to allow for

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biofilm formation, the wells were washed two times with PBS to remove any nonadherent cells.

In the second model, biofilms were grown on silicon elastomer (SE) surfaces (Cardiovascular Instrument Corp., Wakefield, MA) as described previously (25, 31). Briefly, strains were grown overnight in YPD medium at 30°C, washed in PBS, and diluted to a concentration of 5×10^6 cells/ml in RPMI or SU medium. The suspension was added to a sterile 24-well plate containing SE squares (cardiovascular instrument silicone sheets PR72034-06N). The inoculated plates were incubated at 37°C for 90 min for initial adhesion of cells. The squares were washed with PBS, transferred to fresh plates containing the respective medium, and incubated at 37°C for 24 h at 100 rpm to allow biofilm formation.

The extent of biofilm formation was estimated using a semiquantitative colorimetric 2,3-bis(2-methoxy-4-nitro-5-sulphophenyl)-2H-tetrazolium-5-carboxanilide (XTT) reduction assay as reported previously by our group (18, 23). Briefly, XTT (Sigma) was prepared in a saturated solution at 0.5 g/liter in Ringer's lactate. The solution was filter sterilized in a 0.22- μ m-pore-size filter, aliquoted, and stored at -70°C. Prior to each assay, an aliquot of stock XTT was thawed, and menadione (10 mM concentration prepared in acetone; Sigma) was added to a final concentration of 1 μ M. XTT-menadione solution (80 μ l) was added to each prewashed biofilm and control wells (serving as a background measurement). The microtiter plates were incubated in the dark at 37°C for 2 h. Following incubation, 75 μ l of XTT-menadione solution was transferred to the wells of a new microtiter plate, and the colorimetric change from XTT reduction was read in a microtiter plate reader (Benchmark Microplate Reader; Bio-Rad, Hercules, CA) at 490 nm.

Bright-field microscopy. Bright-field light microscopy techniques on an inverted system microscope (Westover Scientific, Mill Creek, WA) equipped for photography were used to routinely monitor biofilm formation. The images were processed for display using Micron software (Westover Scientific).

SEM. Biofilms formed on SE were placed in fixative (4% formaldehyde [vol/vol]–1% glutaraldehyde [vol/vol] in PBS) overnight. The samples were rinsed in 0.1 M phosphate buffer (two times for 3 min each) and then placed in 1% Zetterquist's osmium for 30 min. The samples were subsequently dehydrated in a series of ethanol washes (70% for 10 min, 95% for 10 min, and 100% for 20 min), treated (two times for 5 min each) with hexamethyldisilazane (Polysciences Inc., Warrington, PA), and finally air dried in a desiccator. The specimens were coated with gold and palladium (40%/60%). After the samples were processed, they were observed in a scanning electron microscope ([SEM] Leo 435 VP) in high-vacuum mode at 15 kV. The images were processed for display using Photoshop software (Adobe, Mountain View, CA).

CSLM. *C. albicans* biofilms formed on SE were stained with 25 μ g/ml concanavalin A-Alexa Fluor 594 conjugate (C-11253; Molecular Probes, Eugene, OR) for 1 h in the dark at 37°C. Confocal scanning laser microscopy (CSLM) was performed with a Zeiss LSM 510 upright confocal microscope (Carl Zeiss, Thornwood, NY) using a Zeiss Achroplan 40 $\times$ /0.8 W objective. Concanavalin A conjugate staining was observed using an HeNe1 laser with an excitation wavelength of 543 nm. Images were assembled into side and depth views using the Zeiss LSM Image Browser software, version 4.2. Artificially colored depth view images were used to indicate cell depth using a color gradient wherein cells closest to the SE were represented in blue, and the cells farthest away were represented in red.

RNA extraction and real-time PCR. RNA was extracted from 24-h SE-grown biofilms using a MasterPure Yeast RNA Purification Kit (Epicentre Biotechnologies, Madison, WI). The integrity of the RNA was tested via gel electrophoresis. A total of 1 μ g of RNA was treated with amplification grade DNase I (Invitrogen, Carlsbad, CA) and used for cDNA synthesis with a cMaster RT Kit (Eppendorf AG, Hamburg, Germany) as per the manufacturer's instructions. The following primer sets were used in conjunction with SYBR Green PCR Master Mix (Applied Biosystems, Forest City, CA) and twin.tec real-time 96-well PCR plates (Eppendorf AG, Hamburg, Germany) in an ABI 7300 Real Time PCR System (Applied Biosystems, Forest City, CA): for *NRG1*, CAC CTC ACT TGC AAC CCC and GCC CTG GAG ATG GTC TGA (29); *ACT1*, ATG TGT AAA GCC GGT TTT GCC G and CCA TAT CGT CCC AGT TGG AAA C (29); *HWP1*, TCA GCC TGA TGA CAA TCC TC and GCT GGA GTT GTT GGC TTT TC; *ALS3*, CAA CTT GGG TTA TTG AAA CAA AAA CA and AGA AAC AGA AAC CCA AGA ACA ACC T (13); *EFG1*, GCC TCG AGC ACT TCC ACT GT and TTT TTT CAT CTT CCC ACA TGG TAG T; and *TUPI*, GCT TCA GGT AAC CCA TTG TTG AT and CTT CGG TTC CCT TTG AGT TTA GG. Parameters for primer design were set according to the recommendations of Applied Biosystems. Briefly, the primer sizes were between 20 and 25 bases in length, and the thermal denaturation midpoint temperature of each primer was 58°C. The amplicons were between 90 and 110 bp in size. Each reaction mixture was set up in triplicate in a 25.0- μ l volume with 25 ng of cDNA

FIG. 1. Kinetics of *C. albicans* SC5314 strain biofilm formation in RPMI (■) and SU (▲) media as determined by XTT colorimetric readings. OD, optical density.

for 40 cycles (thermal cycling conditions: initial steps of 50°C for 2 min and 95°C for 10 min, followed by 40 cycles of 95°C for 15 s and 60°C for 1 min). Relative gene expression was quantified using the threshold cycle (C_T) method using the 7300 System Sequence Detection software with the RQ Study Application from Applied Biosystems (32). The target genes were normalized to the housekeeping gene *ACT1*. The relative change in expression was calculated for each sample using the $2^{-\Delta\Delta C_T}$ method, and results from the different replicates were averaged after the $2^{-\Delta\Delta C_T}$ calculations.

In vitro biofilm antifungal susceptibility testing. Fluconazole (Pfizer Inc., New York, NY), amphotericin B (Sigma), and caspofungin (Merck Research Laboratories, Rahway, NJ) were used in the current study. Stock solutions (1 mg/ml) of each individual drug were prepared in dimethyl sulfoxide and stored at 4°C until used.

Biofilms were formed in 96-well microtiter plates with SU or RPMI medium as described above. After biofilm formation for 24 h, the plates were washed, and dilutions of drugs in fresh medium (RPMI medium) were added to the wells containing preformed biofilms. Concentrations of fluconazole (0.25 to 1,024 μ g/ml), amphotericin B (0.25 to 16 μ g/ml), and caspofungin (0.015 to 16 μ g/ml) were examined. The biofilms were then incubated in the presence of the antifungal agent for an additional 24 h. Untreated biofilms containing RPMI medium were included to serve as positive controls. Measurement of biofilm cell metabolic activity using the XTT reduction assay was performed as described above. Assays were performed in triplicate. The antifungal effect was determined by comparing the reduction in mean absorbance of the antifungal-challenged biofilm condition to unchallenged controls, and results are expressed as sessile MIC₅₀ and MIC₈₀ values (SMIC₅₀ and SMIC₈₀, respectively).

RESULTS

Characteristics of *C. albicans* biofilm development in SU medium. We hypothesized that *C. albicans* biofilms formed in the presence of urine may be less robust than biofilms grown in a laboratory medium such as RPMI medium, which is commonly used to form biofilms in vitro. To test this, *C. albicans* biofilms were developed for 48 h in SU and RPMI media on 96-well microtiter plates and SE pieces. Metabolic activity of the biofilm cells was measured by XTT assay at several time points of biofilm formation, and biofilms were also examined by light microscopy. Results revealed that biofilms in the two media had comparable XTT values beyond 24 h. However, cells within the SU medium biofilms appeared to be at least twofold less metabolically active in the first 12 h of biofilm growth than the cells in the RPMI medium biofilm (Fig. 1). Thus, we used light microscopy to monitor biofilm formation and morphology of cells within the biofilms at various points during this period of biofilm development in both media. Results revealed that at 4 h, the cells in RPMI medium biofilms had proliferated and extended long hyphae and pseudohyphae

FIG. 2. Light microscopy images of *C. albicans* SC5314 biofilms in RPMI and SU media. The different panels show photomicrographs taken at various time points during biofilm development, as indicated.

while the SU medium biofilm cells were still in the yeast form and had just begun to develop short germ tubes (Fig. 2). After 8 h in RPMI medium, hyphae from neighboring microcolonies merged into an intricate network of cells composed mostly of filamentous forms. In contrast, biofilms formed in SU medium still contained significant numbers of yeast cells (Fig. 2). As further biofilm maturation occurred over 24 h, biofilm complexity increased significantly, with all fungal morphologies being present in the final biofilm structure in both media. However, the density of cells in the biofilm grown in SU medium was still lower than that observed for RPMI medium-grown biofilms (Fig. 2).

SEM and CSLM visualization of biofilms grown in SU and RPMI media. Architectural features of biofilms grown in the

two media were further studied by SEM and CSLM. For these two microscopic techniques, we developed biofilms on SE pieces for 24 h. Results from SEM revealed that the biofilms in RPMI medium were predominantly made up of an intricate mesh of long hyphae and pseudohyphae (Fig. 3A). On the other hand, biofilms grown in SU medium consisted mostly of elongated yeast cells, pseudohyphae, and short hyphae, with sporadic presence of longer hyphal elements (Fig. 3B). The CSLM images confirmed the SEM results in that the biofilm in SU medium was less complex and five times thinner than biofilms formed in RPMI medium, with a thickness of approximately 300 μm in RPMI and 60 μm in SU (Fig. 4).

Expression profiling of filamentation-associated genes in biofilms grown in SU and RPMI media. Microscopic imaging revealed that biofilms in SU medium contained far fewer hyphae than biofilms in RPMI medium. Since hyphae are important elements providing structural integrity to fully developed biofilms, this observation agrees with the fact that biofilms grown in SU medium are structurally less complex (and thinner) than their RPMI medium-grown counterparts. We questioned if this phenotypic difference was also accompanied by alterations in gene expression, particularly for genes playing important roles in filamentation. Thus, we examined levels of gene expression for five filamentation-associated genes including *ALS3*, *HWP1*, *EFG1*, *NRG1*, and *TUP1* in 24-h biofilms using quantitative reverse transcriptase PCR. The expression of these genes in biofilms from SU medium was compared to that detected in biofilms grown in RPMI medium. We found that levels of gene expression for the positive regulator of filamentation *EFG1* (28) together with *ALS3* and *HWP1*, encoding hyphal-specific adhesins with essential and complementary roles during biofilm formation (15), were 4- to 20-fold less abundant in biofilms from SU medium. At the same time, *NRG1*, a negative regulator of filamentation (3), showed a greater than twofold upregulation in SU medium-formed biofilms. Expression levels of *TUP1* (another, more global, negative regulator of filamentation) (2) showed no significant differences in the two different media (Fig. 5).

Antifungal susceptibility testing of *C. albicans* biofilms formed in SU medium against clinically used antifungal agents. Biofilms were formed on 96-well microtiter plates in both SU and RPMI media. In vitro activity of clinically used fluconazole, amphotericin B, and caspofungin against preformed biofilms formed using both media was assessed using the XTT reduction assay. Similar to observations for RPMI medium, results from these experiments showed the intrinsically high level of resistance to fluconazole of sessile *C. albicans* cells present in biofilms grown in SU medium, as well as increased resistance against amphotericin B (Table 1). Caspofungin was the only antifungal agent that displayed potent in vitro antifungal activity against biofilms in both RPMI and SU media, as indicated by the low SMIC_{50} and SMIC_{80} values (Table 1).

DISCUSSION

Catheter-associated UTIs are the most common nosocomial infection in hospitals and nursing homes, accounting for approximately 40% of nosocomial infections and resulting in increased morbidity, mortality, and length of hospital stay.

FIG. 3. SEM images of mature (24 h) *C. albicans* SC5314 biofilms formed on SE pieces using RPMI or SU medium.

Nosocomial bacteriuria or candiduria may develop in up to 25% of patients requiring a urinary catheter for longer than 7 days (11).

C. albicans biofilms in vitro are developed frequently in nutrient-rich laboratory media. However, under in vivo settings, *C. albicans* biofilms that grow on urinary catheters are intermittently bathed in patient's urine as the only source of nutrient. We hypothesized that *C. albicans* biofilms formed in the presence of SU medium may be less robust than biofilms grown in RPMI medium, the most frequently used medium for the formation of fungal biofilms. Thus, we initiated a study to characterize and compare *C. albicans* biofilms grown in SU and RPMI media. The impact of complications caused by biofilms on patient care and well-being emphasizes the significance of this study.

We found that while *C. albicans* biofilm formation in both RPMI and SU media followed similar developmental stages (adhesion, microcolony formation, filamentation, and prolifer-

ation), cell growth during the initial phases of growth (0 to 20 h) was at least twofold slower in SU medium than in RPMI medium (Fig. 1). In particular, the rate of yeast to hyphal proliferation in SU medium was significantly delayed between 2 to 12 h (Fig. 2). Moreover, SEM and CSLM results revealed that the 24-h *C. albicans* biofilms grown in SU medium were less filamentous and, as a consequence, architecturally less complex than biofilms grown in RPMI medium (Fig. 3 and 4). Thus, overall the decrease in biofilm complexity in SU medium can be attributed to the delay in cell growth and proliferation during the initial phases of biofilm development and, most importantly, to the filamentation defect. This decrease was independent of the adhesive properties of the cells because adhesion assays revealed similar levels of cellular adhesion in SU and RPMI media (not shown).

Our results are somewhat different from those reported by Jain et al., who observed an increase in the amount of biofilm formation by *Candida* clinical isolates in an artificial urine (AU) medium compared to biofilm formation in RPMI medium (7). Differences in composition of the media used in both studies could play a major role in the difference in biofilm biomass quantities. AU medium contains 8% glucose, a concentration fourfold higher than that present in the SU medium used in our studies and severalfold higher than the physiological concentration of glucose present in urine (16). In fact, when Jain et al. increased the concentration of glucose in RPMI medium to 8%, they reported a corresponding increase in biofilm biomass (7).

Since biofilms developed in SU medium showed filamentation defects, we posited that this difference will likely be accompanied by differences in levels of gene expression of key filamentation-associated genes, some of which have also been implicated in biofilm formation. Indeed, we found that levels of expression of *EFG1*, *ALS3*, and *HWPI* in biofilms grown in SU were lower than in biofilms grown in RPMI medium. This is in agreement with previous results by our group and others. We have previously described a pivotal role for Efg1p, a transcriptional regulator of filamentation in *C. albicans* biofilm formation (22). Likewise, Als3p and Hwp1 are two hyphal-specific adhesins that provide complementary cell-to-cell adhesive functions necessary for biofilm integrity (15). On the

FIG. 4. *C. albicans* SC5314 biofilms developed in RPMI and SU media were stained with concanavalin A conjugate for CSLM visualization, and image reconstructions were created to provide side views (A and B). CSLM depth views for the biofilms (C and D) were artificially colored: blue represents cells closest to the silicone, and red represents cells farthest from the silicone (scale is in μm).

FIG. 5. Results of quantitative reverse transcriptase PCR for gene expression levels of five filamentation-associated genes (*ALS3*, *HWP1*, *NRG1*, *EFG1*, and *TUP1*) in *C. albicans* SC5314 biofilms. The figure shows relative differences in expression of the five genes in biofilms grown in SU medium compared to those grown in RPMI medium. Values are means and standard deviations from one experiment performed in triplicate. Experiments were repeated with similar results.

other hand, gene expression levels for *NRG1*, encoding a negative regulator of filamentation, were higher in SU medium-grown biofilms. A major constituent of the defined SU medium is urea, an essential component present in urine. Urea is known to cause cell cycle delays and apoptosis in mammalian cells (12). We speculate that the presence of urea in the medium could be a factor resulting in the delay of the transition of yeast to the hyphal stage.

C. albicans biofilms in vitro as well as in vivo are known to be resistant to all major classes of antifungal drugs except echinocandins and liposomal formulations of amphotericin B (9, 21). Drug resistance has been partially attributed to increased cell density in biofilms (17). We found that despite being architecturally simpler and thinner than RPMI medium biofilms, the SU medium biofilms were still completely resistant to fluconazole and displayed increased resistance to amphotericin B. These results also indicate that biofilm resistance is independent of filamentation, supporting previous observations (22). The only drug which showed potent activity in both SU and RPMI media was caspofungin. This is consistent with several

published reports (1, 5, 9) but is contrary to results reported for AU medium-grown biofilms (7). A caveat here is that urine concentrations achieved by echinocandin antifungal agents are normally low; however, caspofungin has been previously demonstrated to be effective in the treatment of symptomatic candiduria (27).

In summary, *C. albicans* is capable of forming biofilms in an SU-based medium. However, the morphological and architectural characteristics of the resulting biofilms are different from those formed in biofilms using RPMI medium. Caspofungin may represent a useful antifungal for the treatment of recalcitrant UTIs associated with biofilm formation on urinary catheters.

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TABLE 1. Results of antifungal susceptibility testing against *C. albicans* SC5314 biofilms formed using RPMI or SU medium

Antifungal agent	Growth medium	SMIC ₅₀ (μg/ml)	SMIC ₈₀ (μg/ml)
Fluconazole	RPMI	>1,024	>1,024
	SU	>1,024	>1,024
Amphotericin B	RPMI	1	16
	SU	16	>16
Caspofungin	RPMI	0.03125	0.03125
	SU	0.03125	0.03125

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