Biofilm Production and Antibiofilm Activity of Echinocandins and Liposomal Amphotericin B in Echinocandin-Resistant Yeast Species

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The echinocandins and liposomal amphotericin B are active against biofilm produced by echinocandin-susceptible *Candida* strains. However, few data have been reported on the production of biofilm by echinocandin-resistant isolates and their antifungal susceptibility. We studied the production of biofilm by *fks* mutant *Candida* strains and intrinsically echinocandin-resistant non-*Candida* isolates and the susceptibility of both entities to liposomal amphotericin B and echinocandins. We analyzed the production of biofilm by isolates from patients with fungemia (*fks* mutant *Candida*, *n* = 5; intrinsically echinocandin-resistant non-*Candida*, *n* = 12; and *Candida* wild type, *n* = 10). Biofilm formation was measured to classify strains according to biomass (crystal violet assay) and metabolic activity (XTT reduction assay). Preformed biofilms were tested against liposomal amphotericin B, caspofungin, micafungin, and anidulafungin. The sessile MIC was defined as the antifungal concentration yielding a 50% or 80% reduction in the metabolic activity of the biofilm compared to that of the growth control (SMIC₅₀ and SMIC₈₀, respectively). *fks* mutant *Candida* isolates formed biofilms in a fashion similar to that of *Candida* wild-type strains. The echinocandins had the highest activity against biofilms formed by wild-type *Candida* isolates, followed by *fks* mutant *Candida* isolates and non-*Candida* isolates. Liposomal amphotericin B had the highest activity against *fks* mutant *Candida* biofilms. The formation of biofilm by echinocandin-resistant strains was similar to that of wild-type strains, although resistance to echinocandins remained high.

Biofilm production allows *Candida* spp. to attach to catheters and other bioprosthetic devices, leading to catheter-related candidemia and complicating the management of patients with invasive candidiasis (1). *Candida* biofilms are difficult to eradicate because they are resistant to many antifungal agents and host immune mechanisms. In addition, associated infections increase morbidity and mortality rates (1–4). Echinocandins and liposomal amphotericin B are active against biofilm (5, 6), and patients with fungemia receiving these antifungal agents may not need early catheter removal (7).

Echinocandin resistance is increasingly frequent and complicates the management of patients with candidemia caused by *C. glabrata* (8). Resistance to echinocandins in *Candida* spp. is a consequence of mutations in the *fks* gene family (9–11). Other non-*Candida* species, such as *Trichosporon*, *Rhodotorula*, and *Saccharomyces*, rarely cause fungemia, although they are intrinsically resistant to echinocandins. Compared with wild-type *Candida* isolates, *fks* mutant *Candida* isolates and intrinsically echinocandin-resistant non-*Candida* isolates are thought to form biofilms that are highly resistant to echinocandins. In contrast, biofilms may be susceptible to liposomal amphotericin B regardless of their susceptibility to echinocandins. Only two studies have proved that *fks* mutant *Candida* isolates are able to form biofilms; however, very few isolates were tested (12) or only *fks* mutant *C. albicans* isolates were characterized (13). Little is known about the antifungal susceptibility of the biofilms formed by isolates belonging to non-*albicans Candida* species or intrinsically echinocandin-resistant non-*Candida* species to echinocandins or liposomal amphotericin B.

We characterized biofilm production, measured in terms of biomass production and metabolic activity, in clinical wild-type *Candida*, *fks* mutant *Candida*, and intrinsically echinocandin-resistant non-*Candida* strains. We also studied the antifungal susceptibility of these strains to echinocandins and liposomal amphotericin B.

(Results from this study were presented at the 7th Congress on Trends in Medical Mycology in Lisbon, Portugal, 2015 [14].)

MATERIALS AND METHODS

Organisms and identification. We studied 5 previously reported *fks* mutant *Candida* isolates (*Candida albicans*, *n* = 1; *Candida glabrata*, *n* = 1; *Candida tropicalis*, *n* = 3) (15, 16) (Table 1) and 10 wild-type *Candida* species isolates, 2 for each *fks* mutant isolate, matching the biofilm profile and species of the mutant isolates (*C. albicans*, *n* = 2; *C. glabrata*, *n* = 2; *C. tropicalis*, *n* = 6). In addition, we analyzed 12 intrinsically echinocandin-resistant non-*Candida* species (*Rhodotorula mucilaginosa*, *n* = 7; *Tricho-*

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TABLE 1 Formation of biofilm by *fks* mutant *Candida* isolates (*n* = 5) and intrinsically echinocandin-resistant non-*Candida* isolates (*n* = 12)^a

<i>fks</i> mutation or resistance status	Species	Biofilm biomass (classification)	Biofilm metabolic activity (classification)
F641S <i>fks1</i>	<i>C. albicans</i>	1.16 (MBF)	0.19 (MMA)
Δ649 <i>fks2</i>	<i>C. glabrata</i>	0.03 (LBF)	0.20 (HMA)
S645F <i>fks1</i>	<i>C. tropicalis</i>	1.84 (HBF)	0.17 (MMA)
F641L <i>fks1</i>	<i>C. tropicalis</i>	0.77 (MBF)	0.15 (MMA)
R647G <i>fks1</i>	<i>C. tropicalis</i>	2.33 (HBF)	0.14 (MMA)
Wild type	<i>C. albicans</i>	0.47 (MBF)	0.18 (MMA)
Wild type	<i>C. albicans</i>	0.86 (MBF)	0.16 (MMA)
Wild type	<i>C. glabrata</i>	0.16 (LBF)	0.24 (HMA)
Wild type	<i>C. glabrata</i>	0.25 (LBF)	0.21 (HMA)
Wild type	<i>C. tropicalis</i>	0.94 (MBF)	0.13 (MMA)
Wild type	<i>C. tropicalis</i>	1.04 (MBF)	0.10 (MMA)
Wild type	<i>C. tropicalis</i>	1.98 (HBF)	0.17 (MMA)
Wild type	<i>C. tropicalis</i>	1.75 (HBF)	0.16 (MMA)
Wild type	<i>C. tropicalis</i>	1.92 (HBF)	0.14 (MMA)
Wild type	<i>C. tropicalis</i>	2.32 (HBF)	0.18 (MMA)
Intrinsically resistant	<i>A. adeninivorans</i>	0.05 (LBF)	0.01 (LMA)
Intrinsically resistant	<i>R. mucilaginosa</i>	0.05 (LBF)	0.01 (LMA)
Intrinsically resistant	<i>R. mucilaginosa</i>	0.13 (LBF)	0.03 (LMA)
Intrinsically resistant	<i>R. mucilaginosa</i>	0.13 (LBF)	0.03 (LMA)
Intrinsically resistant	<i>R. mucilaginosa</i>	0.06 (LBF)	0.01 (LMA)
Intrinsically resistant	<i>R. mucilaginosa</i>	0.09 (LBF)	0.01 (LMA)
Intrinsically resistant	<i>R. mucilaginosa</i>	0.09 (LBF)	0.01 (LMA)
Intrinsically resistant	<i>R. mucilaginosa</i>	0.06 (LBF)	0.01 (LMA)
Intrinsically resistant	<i>T. asahii</i>	0.11 (LBF)	0.01 (LMA)
Intrinsically resistant	<i>T. asahii</i>	0.08 (LBF)	0.03 (LMA)
Intrinsically resistant	<i>T. dermatis</i>	0.29 (LBF)	0.02 (LMA)
Intrinsically resistant	<i>T. japonicum</i>	0.54 (MBF)	0.13 (MMA)

^a The formation of biofilm by *fks* mutant *Candida* isolates and intrinsically echinocandin-resistant non-*Candida* isolates was studied using biomass production (crystal violet) and biofilm metabolic activity (XTT reduction). The biofilms are classified according to our previously reported scores (17).

sporon asahii, *n* = 2; *Trichosporon japonicum*, *n* = 1; *Trichosporon dermatis*, *n* = 1; *Arxula adeninivorans*, *n* = 1). All isolates were obtained from positive blood cultures of patients with fungemia and were identified using the ID 32C system (bioMérieux, Marcy l’Etoile, France) and confirmed by amplification and sequencing of the ITS1-5.8S-ITS2 region (15, 16).

Biofilm formation. Biofilm was formed according to our previously reported methodology (17). Briefly, a loopful of cultured yeasts was inoculated in 20 ml of yeast-peptone-dextrose broth (Difco, Becton Dickinson, Madrid, Spain) and incubated overnight at 30°C. After centrifugation, cells were resuspended in 20 ml of phosphate-buffered saline (PBS) and centrifuged at 3,000 × *g* for 5 min for washing. This procedure was performed twice, and washed cells were resuspended in 10 ml of RPMI 1640 broth medium. The suspension was adjusted to 1 × 10⁶ cells/ml, 100 μl of the suspension was inoculated in 96-well trays and incubated for 24 h at 37°C, and biofilm was quantified by following two procedures: (i) crystal violet staining to assess biofilm production (18) and (ii) the XTT reduction assay to assess the metabolic activity of the biofilm (19). Strains were tested in triplicate and classified based on our previously reported score (17) (i.e., according to the biomass production obtained by crystal violet assay) as low biofilm forming (LBF; <0.44), moderate biofilm forming (MBF; 0.44 to 1.17), and high biofilm forming (HBF; >1.17). Strains were also classified based on their metabolic activity using the XTT reduction assay as low metabolic activity (LMA; <0.097), moderate metabolic activity (MMA; 0.097 to 0.2), and high metabolic activity (HMA; >0.2).

Antifungal susceptibility. The antifungal activity of anidulafungin (Pfizer Pharmaceutical Group, New York, NY, USA), caspofungin (Merck & Co., Inc., Rahway, NJ, USA), micafungin (Astellas Pharma, Inc., Tokyo, Japan), and liposomal amphotericin B (AmBisome Gilead

Sciences) was assessed against planktonic yeasts and biofilms using the EUCAST EDef 7.2 procedure and the XTT reduction assay, respectively (20, 21). Echinocandins were obtained from standard powders and the concentrations prepared according to the potency of the drug. Liposomal amphotericin B was prepared according to the manufacturer’s instructions. Briefly, 12 ml of distilled water was added to a vial containing 50 mg of liposomal amphotericin B to obtain a solution with an amphotericin B concentration equal to 4,000 μg/ml. The filtered solution was subsequently diluted in distilled water to a final stock solution of 1,280 μg/ml.

Preformed biofilms were treated with increasing 2-fold concentrations of the four drugs, ranging from 0.015 μg/ml to 16 μg/ml, and plates were incubated at 37°C for 24 h. After the antifungal was removed, a solution comprising 0.5 mg/ml XTT and 0.1 mM menadione (Sigma-Aldrich, Madrid, Spain) was added, and the plates were incubated in darkness for 2 h. The XTT solution was read spectrophotometrically at 490 nm (Multiskan FC microplate photometer; Thermo Scientific, Madrid, Spain). The sessile MIC₅₀ (SMIC₅₀) and MIC₈₀ (SMIC₈₀) were defined as a 50% and 80% reduction, respectively, in the metabolic activity of the biofilm treated with the antifungal compared to activity in the control well.

SEM. For scanning electron microscopy (SEM), we studied the biofilm structure of the *fks* mutant *Candida* isolates (*n* = 5), intrinsically echinocandin-resistant non-*Candida* isolates (*n* = 3), and *Candida* wild-type strains (*n* = 4). The wild-type strains were chosen according to the biofilm production profile (biomass production and metabolic activity) that matched the *fks* mutant *Candida* isolates. We also studied the impact of micafungin and liposomal amphotericin B on the biofilm structure.

Biofilms were formed on 50-mm polystyrene discs and prepared for

FIG 1 Biofilm structure of *fks* mutant strains, wild-type isolates with the same biofilm profile, and intrinsically echinocandin-resistant non-*Candida* species. Magnification, $\times 5,000$. (a) *C. glabrata* wild-type, (b) *C. glabrata* $\Delta 649$ *fks2*, (c) *Trichosporon japonicum*, (d) *C. tropicalis* wild-type, (e) *C. tropicalis* S645F *fks1*, (f) *Rhodotorula mucilaginosa*, (g) *C. albicans* wild-type, (h) *C. albicans* F641S *fks1*, (i) *Arxula adeninivorans*, (j) *C. tropicalis* wild-type, (k) *C. tropicalis* F641L *fks1*, (l) *C. tropicalis* wild-type, and (m) *C. tropicalis* R647G *fks1* strains were examined.

TABLE 2 Drug susceptibility of the planktonic form of *Candida* and non-*Candida* isolates

Mutation or <i>in vitro</i> echinocandin resistance status	Species	Planktonic MIC (μg/ml)			
		Caspofungin	Micafungin	Anidulafungin	L-amphotericin B
F641S FKS1	<i>C. albicans</i>	0.5	1	0.25	0.062
Δ649 FKS2	<i>C. glabrata</i>	4	2	1	0.25
S645F FKS1	<i>C. tropicalis</i>	0.5	1	2	0.25
F641L FKS1	<i>C. tropicalis</i>	0.125	0.125	0.062	0.25
R647G FKS1	<i>C. tropicalis</i>	0.125	0.25	0.062	0.25
Wild type	<i>C. albicans</i>	0.125	≤0.015	≤0.015	0.125
Wild type	<i>C. albicans</i>	0.062	≤0.015	≤0.015	0.25
Wild type	<i>C. glabrata</i>	0.125	≤0.015	≤0.015	0.25
Wild type	<i>C. glabrata</i>	0.062	≤0.015	≤0.015	0.5
Wild type	<i>C. tropicalis</i>	0.125	≤0.015	≤0.015	0.25
Wild type	<i>C. tropicalis</i>	0.125	0.031	≤0.015	0.125
Wild type	<i>C. tropicalis</i>	0.125	0.031	0.031	0.25
Wild type	<i>C. tropicalis</i>	0.125	0.031	≤0.015	0.25
Wild type	<i>C. tropicalis</i>	0.125	0.031	≤0.015	0.25
Wild type	<i>C. tropicalis</i>	0.125	≤0.015	≤0.015	0.125
Intrinsically resistant	<i>A. adeninivorans</i>	0.25	0.125	0.5	1
Intrinsically resistant	<i>R. mucilaginosa</i>	2	≥16	≥16	2
Intrinsically resistant	<i>R. mucilaginosa</i>	≥16	≥16	≥16	1
Intrinsically resistant	<i>R. mucilaginosa</i>	≥16	≥16	≥16	0.125
Intrinsically resistant	<i>R. mucilaginosa</i>	≥16	≥16	≥16	0.25
Intrinsically resistant	<i>R. mucilaginosa</i>	≥16	≥16	≥16	0.25
Intrinsically resistant	<i>R. mucilaginosa</i>	1	≥16	≥16	1
Intrinsically resistant	<i>R. mucilaginosa</i>	1	≥16	≥16	0.25
Intrinsically resistant	<i>T. asahii</i>	≥16	≥16	≥16	≥16
Intrinsically resistant	<i>T. asahii</i>	≥16	≥16	≥16	≥16
Intrinsically resistant	<i>T. dermatitis</i>	≥16	≥16	≥16	≥16
Intrinsically resistant	<i>T. japonicum</i>	≥16	≥16	≥16	8

^a We measured the susceptibility to micafungin, caspofungin, anidulafungin, and liposomal amphotericin B (L-amphotericin B) of the planktonic form of the *fk*s mutant *Candida* isolates, wild-type *Candida* isolates, and intrinsically echinocandin-resistant non-*Candida* species.

SEM as previously reported (17). In order to study the antifungal effect, preformed biofilms of each isolate were incubated for 24 h at 37°C with 100 μl of RPMI solution containing a drug concentration equal to the SMIC₈₀. The structure of the treated and nontreated biofilms was visualized using a scanning electron microscope (JEOL-JSM 6400; Jeol, Tokyo, Japan).

Data analysis. The *in vitro* activity of caspofungin, anidulafungin, micafungin, and liposomal amphotericin B against planktonic and sessile forms is shown as MIC₅₀s and MIC₉₀s, geometric means, and MIC ranges. Differences between the four drugs in antifungal activity against the biofilms were assessed and compared using the Friedman test. Differences in the susceptibility of the biofilms formed by all three types of isolates were studied using the Kruskal-Wallis test.

This study was approved by the local ethics committee (Comité Ético de Investigación Clínica del Hospital Gregorio Marañón [CEIC-A1], study number 316/15).

RESULTS AND DISCUSSION

We found that the biofilm structure of *fk*s mutant *Candida* isolates did not differ considerably from that of wild-type *Candida* strains (Fig. 1) (Table 1). Given the importance of glucan synthase in cell wall biosynthesis, *fk*s mutant *Candida* isolates may not be able to form biofilms. However, Vavala and colleagues (12) found that one *C. albicans* *fk*s mutant isolate was more able to form biofilm than the susceptible wild-type strain. Our study confirms these observations and shows that biofilm formation may be more related to the species of *Candida* than to the presence of *fk*s mutations. We previously reported that the pattern of biofilm production is a species-specific feature (17); the degree of biofilm

formation of the *fk*s mutant *Candida* isolates reported here is consistent with the species-specific pattern, with a high proportion of *C. tropicalis* isolates being classified as HBF and MBF and a high proportion of *C. glabrata* isolates classified as HMA (17) (Table 1). Walraven and colleagues (13) demonstrated that biofilm architecture was altered in *Candida* isolates, showing a nucleotide substitution at position S645P of the *fk*s1 gene. The biofilm showed a predominance of cells as well as pseudohyphae and pit-like structures on the surface, suggesting a substantial cell surface defect. We did not find these structures in *C. albicans* isolates with a nucleotide substitution at position F641S of the *fk*s1 gene. Similarly, Walraven and colleagues did not report the presence of these structures in the isolates showing the F641S substitution (13).

Intrinsically echinocandin-resistant non-*Candida* isolates formed biofilms that were mostly LBF (Table 1) with a structure characterized by the absence of hyphae and a low density of cells attached to the plastic surface (Fig. 1). *Trichosporon* spp. biofilm was characterized by the presence of pseudohyphae and arthrospores, whereas *Rhodotorula* spp. biofilm was formed predominantly by blastospores with an elongated oval shape.

The three echinocandins and liposomal amphotericin B previously have shown potent *in vitro* activity against preformed *Candida* biofilms (5, 6, 22), although data regarding antifungal activity against echinocandin-resistant isolates are very limited. We studied susceptibility to echinocandins and liposomal amphotericin B of preformed biofilms of *Candida* with acquired resistance to echi-

TABLE 3 Antifungal susceptibility to echinocandins and liposomal amphotericin B expressed as SMIC₅₀ and SMIC₈₀ of *Candida* and non-*Candida* isolates^a

Drug and MIC	SMIC ₅₀ (in µg/ml)			SMIC ₈₀ (in µg/ml)		
	<i>fks</i> mutant	Wild type	Intrinsically resistant	<i>fks</i> mutant	Wild type	Intrinsically resistant
Micafungin						
GM ^b	0.99	0.02	11.28	18.38	0.09	19.03
MIC ₅₀	2	≤0.015	≥32	≥32	≤0.015	≥32
MIC ₉₀	16	0.125	≥32	≥32	≥32	≥32
Range	≤0.015–16	≤0.015–0.125	0.031–≥32	2–≥32	≤0.015–≥32	0.25–≥32
Anidulafungin						
GM	1.74	0.04	15.05	32	0.28	20.09
MIC ₅₀	2	0.031	≥32	≥32	0.062	≥32
MIC ₉₀	≥32	0.5	≥32	≥32	≥32	≥32
Range	0.062–≥32	≤0.015–0.5	0.062–≥32	≥32–≥32	0.031–≥32	0.125–≥32
Caspofungin						
GM	1.51	0.14	6.71	12.13	0.37	32
MIC ₅₀	2	0.125	≥32	≥32	0.25	≥32
MIC ₉₀	≥32	≥32	≥32	≥32	≥32	≥32
Range	0.062–≥32	≤0.015–≥32	0.125–≥32	0.5–≥32	0.031–≥32	≥32–≥32
Liposomal amphotericin B						
GM	0.14	0.29	0.38	2.64	21.11	5.64
MIC ₅₀	0.062	0.25	0.25	8	≥32	≥32
MIC ₉₀	4	≥32	≥32	≥32	≥32	≥32
Range	0.031–4	0.062–≥32	≤0.015–≥32	0.125–≥32	4–≥32	≤0.015–≥32

^a Antifungal susceptibility to echinocandins and liposomal amphotericin B was expressed as a 50% and 80% reduction in the metabolic activity of the biofilm (SMIC₅₀ and SMIC₈₀, respectively) of the *fks* mutant *Candida* isolates, wild-type *Candida* isolates, and intrinsically echinocandin-resistant non-*Candida* isolates.

^b GM, geometric mean.

nocandins, intrinsically echinocandin-resistant non-*Candida* isolates, and wild-type *Candida* isolates. To the best of our knowledge, this is the first study in which the activity of liposomal amphotericin B against biofilms formed by echinocandin-resistant clinical yeast isolates has been studied. The effect of the antifungal agents was also studied using SEM.

The antifungal activity of micafungin, anidulafungin, caspofungin, and liposomal amphotericin B against planktonic forms is shown in Table 2. The three echinocandins were barely active against the *fks* mutant isolates and intrinsically echinocandin-resistant non-*Candida* isolates. In contrast, liposomal amphotericin B showed high activity against *fks* mutant *Candida* and *Rhodotorula* isolates but not against *Trichosporon* spp. The antifungal susceptibility of the sessile forms is shown in Table 3. Based on the SMIC₅₀, liposomal amphotericin B (0.29 µg/ml) was the most active drug, followed by micafungin (0.67 µg/ml), anidulafungin (1.15 µg/ml), and caspofungin (1.21 µg/ml) ($P < 0.001$). However, when a more stringent endpoint (SMIC₈₀) was applied, micafungin (2.63 µg/ml) was the most active drug, followed by anidulafungin (4.49 µg/ml), caspofungin (5.13 µg/ml), and liposomal amphotericin B (7.98 µg/ml) ($P < 0.001$). We compared the susceptibility of *fks* mutant *Candida* isolates, wild-type *Candida* isolates, and intrinsically echinocandin-resistant non-*Candida* isolates to the echinocandins and liposomal amphotericin B. Wild-type *Candida* isolates were the most susceptible to the three echinocandins ($P < 0.001$), whereas the *fks* mutant *Candida* isolates were the most susceptible to liposomal amphotericin B, regardless of the endpoint used

(SMIC₅₀ or SMIC₈₀), although the differences were not statistically significant (Table 3).

We showed that *fks* mutant *Candida* isolates, in both the planktonic and the sessile state, were less susceptible to echinocandins than wild-type isolates. Anidulafungin has been reported to be the most active echinocandin against *fks* mutant *Candida* isolates when the SMIC₈₀ is applied (13); however, we found that caspofungin was the most active echinocandin drug for this endpoint. Antifungal susceptibility to echinocandins has been reported to vary depending on the *fks* mutation (23), which can also have an impact on biofilm susceptibility. We aimed to study the anti-biofilm activity of the three echinocandins used in clinical practice. However, *in vitro* antifungal activity of caspofungin should be interpreted carefully because of the interlaboratory variations reported when using this drug (24).

Liposomal amphotericin B had the highest activity against biofilms formed by isolates with acquired resistance (the P value was not significant) or intrinsic resistance ($P < 0.05$) to echinocandins; this finding is consistent with other studies, where conventional amphotericin B had high anti-biofilm activity, even against non-*Candida* isolates (25–29). However, we observed that biofilms formed by *C. tropicalis* isolates were highly resistant to liposomal amphotericin B, which was unable to achieve an 80% reduction in the metabolic activity of the biofilm. When wild-type *C. tropicalis* isolates were removed from the analysis, the geometric mean (range) of the liposomal amphotericin B SMIC₅₀ and SMIC₈₀ was 0.015 µg/ml (0.062 to 0.25 µg/ml) and 11.31 µg/ml (4

FIG 2 SEM showing the structure of biofilms formed by *fks* mutant strains and wild-type controls after treatment with a concentration of liposomal amphotericin equal to the $SMIC_{80}$ of each isolate. Magnification, $\times 2,000$. (a) *C. tropicalis* F641L *fks1* (growth control, $SMIC_{80}$ of 16 $\mu\text{g/ml}$ of liposomal amphotericin B), (b) *C. tropicalis* wild-type (growth control, $SMIC_{80}$ of 16 $\mu\text{g/ml}$ of liposomal amphotericin B), (c) *C. tropicalis* R647G *fks1* (growth control, $SMIC_{80}$ of 8 $\mu\text{g/ml}$ of liposomal amphotericin B), (d) *C. tropicalis* wild-type (growth control, $SMIC_{80}$ of 16 $\mu\text{g/ml}$ of liposomal amphotericin B), (e) *C. albicans* F641S *fks1* (growth control, $SMIC_{80}$ of 0.125 $\mu\text{g/ml}$ of liposomal amphotericin B), (f) *C. albicans* wild-type (growth control, $SMIC_{80}$ of 16 $\mu\text{g/ml}$ of liposomal amphotericin B), (g) *C. glabrata* $\Delta 649$ *fks2* (growth control, $SMIC_{80}$ of 0.125 $\mu\text{g/ml}$ of liposomal amphotericin B), (h) *C. glabrata* wild-type (growth control, $SMIC_{80}$ of 4 $\mu\text{g/ml}$ of liposomal amphotericin B), (i) *C. tropicalis* S645F *fks1* (growth control, $SMIC_{80}$ of 16 $\mu\text{g/ml}$ of liposomal amphotericin B), and (j) *C. tropicalis* wild-type (growth control, $SMIC_{80}$ of 16 $\mu\text{g/ml}$ of liposomal amphotericin B) strains were examined.

to 32 $\mu\text{g/ml}$), respectively. The explanation for this finding is unknown and warrants further investigation.

SEM was used to investigate the effect of liposomal amphotericin B and micafungin on the preformed *Candida* biofilms (Fig. 2 and 3). Liposomal amphotericin B induced subtle changes in *C. albicans* (both wild-type and *fks* mutant biofilms) with the presence of shriveled hyphae and a slightly less dense structure. The antifungal effect on *C. glabrata* biofilms consisted of a prominent decrease in the number of blastospores (in both wild-type and *fks* mutant isolates). Finally, five *C. tropicalis* isolates were studied using SEM (three were HBF [R647G and S645F *fks1* mutant isolates and the corresponding wild-type isolate], and two were MBF

[F641L *fks1* mutant isolate and the corresponding wild-type isolate]); liposomal amphotericin B had a limited impact on the structure of HBF biofilms, whereas it led to shriveled hyphae and a less dense structure in MBF biofilms. The impact of micafungin on preformed biofilms differed between *Candida* wild-type and *fks* mutant isolates: biofilms formed by wild-type strains were moderately affected, whereas those formed by *fks* mutant isolates remained unaffected, with the exception of the *C. glabrata* $\Delta 649$ *fks2* strain, which showed a slight decrease in biofilm density.

Our study is subject to two limitations. First, we only included a small set of *fks* mutant isolates. Second, we did not analyze the

FIG 3 SEM showing the structure of biofilms formed by *fks* mutant strains and wild-type controls after treatment with a concentration of micafungin equal to the $SMIC_{80}$ of each isolate. Magnification, $\times 2,000$. (a) *C. tropicalis* F641L *fks1* (growth control, $SMIC_{80}$ of 16 $\mu\text{g/ml}$ of micafungin), (b) *C. tropicalis* wild-type (growth control, $SMIC_{80}$ of 0.0015 $\mu\text{g/ml}$ of micafungin), (c) *C. tropicalis* R647G *fks1* (growth control, $SMIC_{80}$ of 16 $\mu\text{g/ml}$ of micafungin), (d) *C. tropicalis* wild-type (growth control, $SMIC_{80}$ of 0.031 $\mu\text{g/ml}$ of micafungin), (e) *C. albicans* F641S *fks1* (growth control, $SMIC_{80}$ of 16 $\mu\text{g/ml}$ of micafungin), (f) *C. albicans* wild-type (growth control, $SMIC_{80}$ of 8 $\mu\text{g/ml}$ of micafungin), (g) *C. glabrata* $\Delta 649$ *fks2* (growth control, $SMIC_{80}$ of 2 $\mu\text{g/ml}$ of micafungin), (h) *C. glabrata* wild-type (growth control, $SMIC_{80}$ of 0.015 $\mu\text{g/ml}$ of micafungin), (i) *C. tropicalis* S645F *fks1* (growth control, $SMIC_{80}$ of 16 $\mu\text{g/ml}$ of micafungin), and (j) *C. tropicalis* wild-type (growth control, $SMIC_{80}$ of 0.031 $\mu\text{g/ml}$ of micafungin) strains were examined.

mechanism of resistance of *C. tropicalis* to liposomal amphotericin B, which should be evaluated in future studies.

In conclusion, we showed that the biofilm produced by *fks* mutant *Candida* isolates is similar to that produced by wild-type *Candida* isolates. Liposomal amphotericin B had the highest activity against *fks* mutant *Candida* biofilms and intrinsically resistant non-*Candida* species, with the exception of *C. tropicalis*.

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