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Target Enzyme Mutations Confer Differential Echinocandin Susceptibilities in *Candida kefyr*

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***Candida kefyr* is an increasingly reported pathogen in patients with hematologic malignancies. We studied a series of bloodstream isolates that exhibited reduced echinocandin susceptibilities (RES). Clinical and surveillance isolates were tested for susceptibilities to all three echinocandins, and those isolates displaying RES to one or more echinocandins were selected for molecular and biochemical studies. The isolates were analyzed for genetic similarities, and a subset was analyzed for mutations in the echinocandin target gene *FKS1* and glucan synthase echinocandin sensitivities using biochemical methods. The molecular typing did not indicate strong genetic relatedness among the isolates except for a series of strains recovered from a single patient. Two unrelated isolates with RES had previously uncharacterized *FKS1* mutations: R647G and deletion of amino acid 641 (F641Δ). Biochemical analysis of the semipurified R647G glucan synthase generated differential echinocandin sensitivity (resistance to micafungin only), while the deletion of F641 resulted in a glucan synthase highly insensitive to all three echinocandins. The consecutive isolates from a single patient with RES all harbored the common S645P mutation, which conferred resistance to all three echinocandins. The MIC values paralleled the glucan synthase inhibition kinetic data, although the S645P isolates displayed relatively higher susceptibility to caspofungin (2 μg/ml) than the other two echinocandins (>8 μg/ml). These findings highlight novel and common *FKS1* mutations in *C. kefyr* isolates. The observation of differential susceptibilities to echinocandins may provide important mechanistic insights for echinocandin antifungals.**

Candida kefyr (teleomorph: *Kluyveromyces marxianus*) has been reported as a rare but potentially increasing cause of invasive candidiasis (IC) (1–3), especially in patients with hematologic malignancies (2, 4–6). Isolates of this species have been noted to develop reduced susceptibility to echinocandins, although the majority examined in population analyses remain susceptible (7).

Decreased susceptibility to echinocandins is associated with mutations and polymorphic changes in *FKS1*, the gene that encodes the target enzyme, β-1,3-D-glucan synthase (Fks1p) (8–14). Fks1p is a plasma membrane protein with several regions exposed to the environment on the outer leaflet of the membrane phospholipid layer (13, 15). The catalytic Fks subunits (Fks1p, Fks2p, and Fks3p) together with the regulatory subunit, Rho1p, compose the glucan synthase (GS) complex (16, 17). Reduced echinocandin susceptibilities (RES) are associated with amino acid alterations at mainly two regions or hot spots (HS) of Fks1p (Fksp2 in *Candida glabrata*). Both of these regions (HS1 and HS2) are highly conserved and thought to be important for the interaction of the enzyme with echinocandins, although the mechanism of resistance remains unclear (13, 18). A third region of *FKS1*, HS3, has also been shown to affect the susceptibility to echinocandins (15, 18). HS3 maps downstream and near HS1 and also codes for the amino acids predicted to reside in an environment-exposed region of Fks1p.

We recently observed an increase in the number of *C. kefyr* IC cases in patients with hematologic malignancies at the John Hopkins Hospital (JHH). This observation prompted a retrospective review of all *C. kefyr* infections in two major hematologic wards between 2004 and 2010 (19). Notable findings of that study included a striking seasonality in isolate recovery, with high rates during summer months. The observation of reduced echinocandin antifungal susceptibilities prompted this detailed study of mechanisms.

MATERIALS AND METHODS

***C. kefyr* clinical isolates.** The study was approved by the JHH institutional review board (IRB). All available *C. kefyr* clinical isolates from patients between 1 January 2009 and 31 December 2012 were recovered from the JHH mycology laboratory. Patients with hematologic malignancies receiving intensive chemotherapy at JHH have fungal surveillance cultures (FSC) of throat and rectal swabs or stool specimens performed systematically on admission and weekly thereafter until their discharge. Clinical data, including demographics, the type of the underlying hematologic malignancy, the treatment regimen, and the administration of antifungal agents within 30 days prior to the first positive *C. kefyr* isolate, were summarized.

Other strains and media. *C. kefyr* type strain ATCC 4922 (American Type Culture Collection, Manassas, VA), isolated from buttermilk (20), was used as an unrelated control for genotyping and echinocandin MIC determinations. *Candida albicans* clinical isolate SC5314 (21) was used as an outlier control. All yeasts were routinely maintained or propagated on yeast extract-peptone-dextrose (YPD) liquid or agar plate medium (1% yeast extract, 2% peptone, and 2% dextrose) and grown at 30°C.

Echinocandin susceptibility testing. All strains were grown on Sabouraud dextrose agar at 35°C for 24 h prior to testing for echinocandin susceptibilities by the broth microdilution assay as described by Clinical and Laboratory Standards Institute (CLSI) document M27-A3 (22) (con-

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centration range, 0.015 to 8.0 μg/ml). Echinocandins were obtained from their respective manufacturers (caspofungin, Merck & Co., Rahway, NJ; micafungin, Astellas Pharma Inc., Deerfield, IL; anidulafungin, Pfizer, Inc., New York, NY) and suspended in dimethyl sulfoxide (DMSO) (Sigma, St. Louis, MO) prior to dilution into RPMI medium for MIC assays. The MIC value was determined as the concentration that produced a prominent reduction in turbidity (≥50% reduction in growth) at 24 h. The strains were tested at least twice on different days. Since we repeatedly observed elevated MICs above the published epidemiological cutoff values for *C. kefyr* (23), the modal MICs were calculated using strains ATCC 4922, C115, C117, and C130 as references for the remaining clinical isolates. The strains were chosen because these harbored *FKS* HS amino acid sequences (see below) of known susceptible *C. albicans* (12, 13) and *C. kefyr* (14) strains. The modal MIC values were derived from 2 to 10 measurements per isolate.

***C. kefyr* genotyping.** A repetitive sequence-based PCR method developed for *Candida rugosa* (24) was adapted to fingerprint the *C. kefyr* clinical isolates. Genomic DNA was prepared, using a MasterPure yeast DNA purification kit (Epicentre Biotechnologies, Madison, WI), from *Candida* spp. grown for ~18 h at 30°C in YPD broth cultures with shaking. Oligonucleotides Ca-21 (5′-CATCTGTGGTGGAAAGTAAAC-3′) and Ca-22 (5′-ATAATGCTCAAAGGTGGTAAG-3′) (24) were used at 1.0 μM in a PCR volume of 25 μl containing 100 ng of genomic DNA, using a TaKaRa *Ex Taq* kit (Clontech, Mountain View, CA) with the cold start method as per the manufacturer's recommendation. PCR conditions were initial denaturation at 94°C for 5 min, followed by 35 cycles with a ramping temperature rate of 1.5°C/s for denaturation at 94°C for 15 s, annealing at 51°C for 30 s, and extension at 72°C for 30 s, and a final extension step at 72°C for 5 min. The amplicons were resolved in 6% acrylamide Tris-borate-EDTA (TBE) gels (Invitrogen/Life Technologies Corporation, Grand Island, NY), stained with ethidium bromide, and visualized under UV light. DNA amplicon banding images were captured with a UVP GelDoc-It imaging system, and the data were imported into PyElph, an open-source software tool (sourceforge.net/projects/pyelph/files/releases/) (25) to generate dendrograms, using the unweighted-pair group method of arithmetic mean (UPGMA).

***FKS1* HS sequencing.** HS1 and HS2 were amplified from *C. kefyr* genomic DNA using *C. albicans FKS1* oligonucleotides FKS1-HS1F (5′-ATGGGCTGGTGCTCAACAT-3′), FKS1-HS1R (5′-CCTTCAATTTCAGATGGAACTTGATG-3′), FKS1-HS2F (5′-AAGATTGGTGCTGGTATGGG-3′), and FKS1-HS2R (5′-TAATGGTGCTTGCCAATGAG-3′), as described by Garcia-Effron et al. (26). Each sample reaction mixture contained 0.5 μM primers and 200 ng of genomic DNA in a 25-μl volume and was performed using a JumpStart REDTaq kit (Sigma, St. Louis, MO). The PCR conditions were initial denaturation at 94°C for 2 min, followed by 35 cycles of denaturation at 94°C for 1 min, annealing at 55°C for 2 min, extension at 72°C for 3 min, and final extension at 72°C for 5 min. The amplicons were column purified (Qiagen Sciences Inc., Germantown, MD) and sequenced using an AB3730XL DNA analyzer (AB Biosystems, Fremont, CA) at the DNA Analysis Facility of Johns Hopkins University (JHU). Multiple sequence alignments were conducted using ClustalW and T-Coffee (MacVector v. 12.6.0; MacVector, Inc., Cary, NC), while pairwise sequence alignments were done with EMBOSS Matcher (www.ebi.ac.uk).

Glucan synthase echinocandin inhibition assays. The strains were grown with vigorous shaking at 37°C to the early stationary phase in modified YPD (2% yeast extract, 4% Bacto Peptone, and 4% dextrose) broth, and cells were collected by centrifugation. Cell disruption, membrane protein extraction, and partial 1,3-β-D-glucan synthase purification by-product entrapment were performed as previously described (27). The sensitivity to echinocandin drugs was measured in a polymerization assay using a 96-well multiscreen HTS filtration system (Millipore Corporation, Bedford, MA) in a final volume of 100 μl, as previously described (11). Serial dilutions of the three echinocandin drugs (0.01 to 10,000 ng/ml) were used to determine the inhibition kinetics yielding 50% inhibitory concentration (IC_{50}) values. Control reactions were performed in the presence of 1% DMSO. The reactions were initiated by the addition of partially purified glucan synthase. Inhibition profiles and IC_{50}s were determined using a sigmoidal response (variable-slope) curve-fitting algorithm or two-site competition fitting algorithm with GraphPad Prism software (v. 4.0; GraphPad Software, Irvine, CA).

Nucleotide sequence accession numbers. The partial *FKS* nucleotide sequence data for each isolate were deposited at GenBank (www.ncbi.nlm.nih.gov/GenBank/) and assigned the following accession numbers (in parentheses, HS1 and HS2, respectively): ATCC 4922 (KJ685779 and KJ685792), C113 (KJ685780 and KJ685793), C114 (KJ685781 and KJ685794), C115 (KJ685782 and KJ685795), C116 (KJ685783 and KJ685796), C117 (KJ685784 and KJ685797), C130 (KJ685785 and KJ685798), C131 (KJ685786 and KJ685799), C132 (KJ685787 and KJ685800), C133 (KJ685788 and KJ685801), C134 (KJ685789 and KJ685802), C135 (KJ685790 and KJ685803), and C136 (KJ685791 and KJ685804).

RESULTS

Reduced echinocandin susceptibilities in *C. kefyr* isolates. Twenty-five *C. kefyr* isolates were recovered from 17 patients (Table 1): 11 blood and 14 FSC isolates from 7 and 14 patients, respectively. One patient (number 17) had 7 sequential isolates retrieved from blood ($n = 4$) and stool ($n = 3$) cultures. All patients with *C. kefyr* IC had an underlying diagnosis of acute myelogenous leukemia (AML) and had received prior treatment with multiple antifungal agents. In 6 of 7 (86%) patients with candidemia, *C. kefyr* isolates had elevated MICs to one or multiple echinocandins (relative to susceptible stains; see modal MIC values in Table 1). In 2 patients, bloodstream isolates were resistant to all echinocandins, whereas 1 patient had a *C. kefyr* isolate (C113) that displayed differential susceptibilities to the echinocandins (micafungin MIC, 4.0 μg/ml; caspofungin MIC, 0.125 μg/ml; and anidulafungin MIC, 0.25 μg/ml). All 3 patients had received micafungin (100 mg/day) as the primary prophylaxis or empirical treatment within 30 days prior to a positive culture (median, 14 days; range, 8 to 20 days). In contrast, only 2 of the remaining 4 patients with IC who had low echinocandin MICs had been preexposed to micafungin.

There were 10 patients with *C. kefyr*-positive FSC with no evidence of IC. All but 2 of these isolates displayed MICs within one dilution of the epidemiological cutoff values for all echinocandins (0.25 μg/ml, 0.03 μg/ml, and 0.125 μg/ml for anidulafungin, caspofungin, and micafungin, respectively) (7). Notably, two patients (numbers 1 and 2), had *C. kefyr* isolates that had relatively high MICs to micafungin while those to anidulafungin and caspofungin remained relatively low. Patient number 17 had multiple stool isolates positive for *C. kefyr*: the first two were susceptible to all echinocandins, while the last one, recovered late during his treatment course, had high echinocandin MICs (Table 1 and details below).

Isolate genotyping and *C. kefyr FKS1* analysis. All isolates from different patients were DNA typed and found to share little genetic similarity (Fig. 1). However, sequential isolates recovered from stool and bloodstream cultures from patient 17 shared strong genetic relatedness. Individual bloodstream isolates with RES (C113 and C116), and sequential related isolates recovered from patient 17 were chosen for detailed study.

The *C. kefyr FKS1* homolog (*CkFKS1*) HS1, HS2, and HS3 regions were amplified and sequenced, using primers to *C. albicans FKS1* sequences (26). Alignment of the translated sequences against the corresponding regions of echinocandin-sensitive *C. albicans* (SC5314) (21), two other echinocandin-sensitive *C. kefyr*

TABLE 1 JHH *Candida kefyr* isolates and echinocandin susceptibilities

Patient no.	Hematologic malignancy[a]	Chemotherapy[b]	Isolate	Source	Date of culture (mo and yr)	Treatment with antifungals ≤30 days prior to positive culture (days)[c]				MIC[d]		
						MICA	FLU	VOR	LAMB	ANID	CAS	MICA
1	AML (new)	FLAM	C113	Blood	Jul 2009	8	No	No	No	0.25	0.125	4
2	AML (new)	AcDVP16	C124	Mucosa	Jul 2009	20	No	No	No	0.5	0.125	2
3	MM (relapse)	Other[e]	C125	Mucosa	Jul 2009	No	No	No	No	0.06	0.06	0.03
4	AML (new)	AcDVP16	C126	Mucosa	Jul 2009	11	No	No	No	0.06	0.06	0.25
5	AML (new)	FLAM	C127	Mucosa	Jul 2009	1	No	No	9	0.06	0.03	0.25
6	Pre-B cell ALL	Other[f]	C128	Mucosa	Jul 2009	4	No	No	No	0.06	0.06	0.25
7	AML (new)	None	C129	Mucosa	Jul 2009	6	No	No	No	0.06	0.06	0.25
8	AML (relapse)	Other[g]	C112	Blood	Aug 2009	No	No	No	No	0.03	0.06	0.25
			C115	Blood	Jul 2010	No	No	No	No	0.25	0.25	0.5
9	AML (new)	FLAM	C123	Mucosa	Aug 2009	10	No	7	No	0.5	0.125	1
10	AML (relapse)	FLAM	C117	Blood	Oct 2009	No	10	No	No	0.06	0.06	0.25
11	AML (new)	FLAM	C122	Mucosa	Dec 2009	No	No	2	14	0.06	0.06	0.125
12	AML (new)	AcDVP16	C116	Blood	Aug 2010	20	5	2	No	>8.0	4	>8.0
13	AML (relapse)	FLAM	C119	Mucosa	Sep 2010	No	No	8	21	0.06	0.06	0.125
			C120	Mucosa	Sep 2010	8	No	3	30	0.06	0.06	0.125
14	GS (relapse)	Other[g]	C121	Mucosa	Sep 2010	12	No	11	No	0.06	0.06	0.125
15	AML (relapse)	AcDVP16	C118	Blood	May 2011	14	No	No	23	0.25	0.25	0.5
16	AML (relapse)	Other[h]	C114	Blood	Feb 2010	18	7	No	9	0.5	0.25	1
17	AML (relapse)	AcDVP16	C130	Stool	Oct 2012	1	No	No	No	0.03	0.06	0.25
			C131	Stool	Oct 2012	8	No	No	No	1	0.25	1
			C132	Blood	Oct 2012	14	No	No	No	>8.0	2	>8.0
			C133	Blood	Oct 2012	14	No	No	No	>8.0	2	>8.0
			C134	Blood	Oct 2012	15	No	No	No	>8.0	2	>8.0
			C135	Blood	Oct 2012	16	No	No	No	>8.0	2	>8.0
			C136	Stool	Oct 2012	17	No	No	No	>8.0	2	>8.0
			C. kefyr[i]							0.125	0.25	0.125
			Wild-type MICs[j]							0.06	0.125	0.25

[a] AML, acute myelogenous leukemia; MM, multiple myeloma; ALL, acute lymphocytic leukemia; GS, granulocytic sarcoma.
[b] FLAM, flavopiridol, cytarabine, and mitoxantrone; AcDVP16, cytarabine, daunorubicin, and etoposide.
[c] MICA, micafungin; FLU, fluconazole; VOR, voriconazole; LAMB, liposomal amphotericin B.
[d] ANID, anidulafungin; CAS, caspofungin.
[e] Other: bortezomib, cyclophosphamide, and lenolidomide.
[f] Other: polyethylene glycol (PEG)-asparaginase.
[g] Other: Investigational agent, topotecan, and carboplatin.
[h] Other: Chk-1 inhibitor and clofarabine.
[i] *C. kefyr* ATCC-4922.
[j] Modal values were calculated using MICs observed for *C. kefyr* isolates ATCC-4922, C115, C117, and C130. See Materials and Methods.

blood isolates, and a reference *C. kefyr* ATCC strain (ATCC 4922) revealed amino acid changes in HS1. Isolate C113 had an amino acid change at position 647 (R647G; amino acid positions are relative to *C. albicans* Fksp1), while C116 was missing the codon for amino acid F641 (F641Δ) (Fig. 2). Both DNA strands of the C116 HS1 were sequenced and were in 100% agreement. C113 and C116 had amino acid sequences identical to those of echinocandin-sensitive *C. kefyr* strains at HS2 and HS3 (18; data not shown).

Isolates C113 and C116 were evaluated for *in vitro* GS inhibition with all three echinocandins (11) relative to that for the echinocandin-sensitive C117 isolate (Fig. 3). The GS from the control echinocandin sensitive C117 isolate showed characteristic inhibitory kinetic profiles with 50% inhibitory concentrations (IC_{50s}) of 107.1, 5.4, and 11.8 ng/ml for anidulafungin, caspofungin, and micafungin, respectively. Resistant isolate C113 showed a differential GS inhibition profile with IC_{50}s of 790.9, 33.11, and >10,000 ng/ml for anidulafungin, caspofungin, and micafungin, respectively. These IC_{50}s corresponded to the relatively low MICs to anidulafungin and caspofungin but high MIC to micafungin (Table 1). The GS from the F641Δ strain C116, which exhibited high MICs to all echinocandins, appeared insensitive to inhibition at the highest level of drug (10,000 ng/ml) tested.

Analysis of sequential FSC and bloodstream isolates. Isolates C130 to C136 recovered sequentially from stool and bloodstream cultures from patient 17 were further investigated. Isolates C130 and C133 were first recovered in the patient's stool and blood cultures 4 and 12 days, respectively, after initiation of micafungin (Fig. 4A). The repetitive PCR (Rep-PCR) results suggested that all isolates but one were closely related to the original stool isolate C130 (Fig. 1 and 4B). This dominant genotype (C130) strain that originated from the gastrointestinal tract demonstrated elevated MICs to all echinocandins within a week and was found in the bloodstream in 12 days. The same dominant genotype was repeatedly recovered from the patient's blood cultures on days 12, 13, and 14 and from his stool culture on day 17, 2 days after stopping micafungin. A second unrelated genotype (C132) was recovered from the same blood

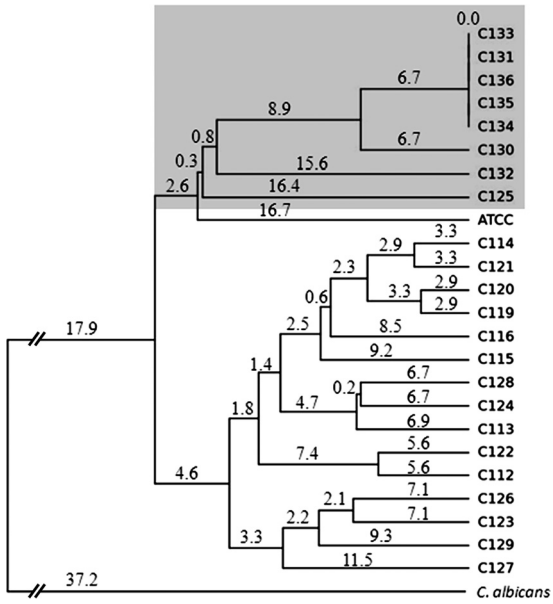

FIG 1 Genetic relatedness of *C. kefyr* isolates shown by a dendrogram and tree analysis of all *C. kefyr* isolates. Genetic relatedness was assessed from the Rep-PCR amplicon patterns using the unweighted-pair group method with arithmetic mean (UPGMA) (35) generated by PyElph software. The genetic distances are shown above the branches. The serial isolates recovered from a single patient are highlighted within a gray shaded box. ATCC, *C. kefyr* ATCC 4922.

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                                    640    #
SC5314     1 DMWMSYLLWFLVFLAKLVESYFFLTLSLRDPIRNLSTMTMRCVGEVWYKD 50
ATCC-4922  1 DMWMSYLMWTTVFAAKYAESYFFLTLSLRDPIRILSTTVMRCTGDYGYKD 50
C113       1 DMWMSYLMVTTVFAAKYAESYFFLTLSLGDPIRILSTTVMRCTGDYGYKD 50
C115       1 DMWMSYLMWTTVFAAKYAESYFFLTLSLRDPIRILSTTVMRCTGDYGYKD 50
C116       1 DMWMSYLMWTTVFAAKYAESYF-LTLSLRDPIRILSTTVMRCTGDYGYKD 49
C117       1 DMWMSYLMWTTVFAAKYAESYFFLTLSLRDPIRILSTTVMRCTGDYGYKD 50
             *******    ** **  **** ***** **** ***  *** *   ***
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FIG 2 Sequence analysis of *C. kefyr FKS1* HS1 from two unrelated blood isolates. Two echinocandin-sensitive isolates, C115 and C117, were used as controls. The sequences were aligned (36, 37) to the homologous *FKS1* region from an irrelevant *C. kefyr* (ATCC 4922) strain and to *C. albicans* (SC5314). The amino acid changes in C113 and C116 are shaded in gray. The most common mutation to confer echinocandin resistance occurs at S645 (11, 13), indicated by a number sign (#) for reference. The amino acid residue numbering (above) is relative to *C. albicans* Fks1p (Gsc1p/Orf19.2929p) (*Candida* Genome Database, http://www.candidagenome.org/).

culture on the same day as the dominant strain (C133), and displayed RES to all echinocandin drugs as well.

Hot spot regions (HS1, HS2, and HS3) of the *CkFKS1* gene from all of these isolates were evaluated by DNA sequencing. A mutation encoding a S645P amino acid change in HS1 of the RES isolates first recovered from the blood was noted and persisted through isolates C132 to C136 (Fig. 5A). No other mutations were noted in HS1, HS2, or HS3 (data not shown). S645P corresponds as well with the most frequent substitution associated with RES in *C. albicans* and *C. glabrata* (13). Although species-specific breakpoints are not clear for *C. kefyr*, the S645P amino acid substitution appeared to generate a GS enzyme complex that is highly insensitive to all three echinocandins (Fig. 5B). However, it was not possible to obtain in-range IC_{50}s, as the sigmoidal dose-response curves did not converge similarly to that observed for a true susceptible isolate (C117) (Fig. 3). Thus, high concentrations of all three echinocandins affected the extracted GS enzyme complex similarly, suggesting that differential susceptibilities measured in *in vitro* testing may reflect a cumulative cellular phenotype.

DISCUSSION

Candida kefyr has been reported as a pathogen of increased concern, especially among people with hematologic malignancies (2, 3). Our center's experience over the last 5 years confirmed an increase in isolation of this species from patients with severe mucositis and neutropenia (19). Data generated in this analysis of FSC and bloodstream isolates reveal two important findings: (i) *FKS1* HS1 mutations in *C. kefyr* are consistent with those in other *Candida* species that generate high-level echinocandin resistance, and these mutations render the GS enzyme complex as relatively resistant to drug inhibition *in vitro*; and (ii) a novel HS1 mutation confers relative resistance of *C. kefyr* to micafungin but preserves activity of the other echinocandin drugs. Testing of enzyme inhibition confirmed that this mutation reduces susceptibility at the level of the GS enzyme complex.

A survey of non-*albicans Candida* collected between 2001 and 2010, as part of the ARTEMIS Global Antifungal Susceptibility Program and the SENTRY Antimicrobial Surveillance Program, a multicenter and multicountry repository of fungal species, found *C. kefyr* isolates susceptible to all three echinocandins (23). However, echinocandin resistance within 10 days of treatment with caspofungin was recently reported for an echinocandin-susceptible *C. kefyr* blood isolate from a patient with AML (14). In our series, echinocandin resistance was observed in 86% of patients who developed IC, all during treatment for AML. Analysis of sequential isolates suggests that the mechanism of IC in these patients is via translocation through the gastrointestinal tract. It is likely that these patients are at particularly high risk due to prolonged neutropenia and severe mucositis, with extensive exposure to antifungals administered in a preventative or empirical fashion. Development of colonization resistance as a prerequisite for bloodstream invasion is suggested by the observation that bloodstream but not mucosal isolates exhibit high MICs in patients who had prolonged exposure to micafungin.

All of our RES *C. kefyr* isolates had amino acid changes in the echinocandin target Fks1p in the region encoded by HS1. Echinocandins exert their action by inhibiting the biosynthesis of the major fungal cell wall component, β-1,3-D-glucan, by interfering with the activity of the catalytic subunit of GS, Fks1p. How these antifungals interact and inhibit Fks1p activity is not entirely understood (13, 15). Although other cellular factors unrelated to Fks1p have been implicated in reduced susceptibility in some *Candida* species, in some *Aspergillus* isolates (28–31), clinical isolates of otherwise susceptible strains such as *C. albicans* and *C. glabrata* have been noted to harbor amino acid changes in Fks1p and Fks2p, respectively. All of the Fks1p homologous regions encompassed by HS1, HS2, and HS3 from different fungi are predicted to reside in a conserved domain of the enzyme embedded in the outer leaflet of the plasma membrane (15).

The molecular and biochemical analyses of *C. kefyr* isolates demonstrate similarities and differences in the way that echinocandins inhibit β-glucan synthesis. Substitution of F641, which is conserved in diverse yeasts and molds, has been reported in up to 33% of RES *C. albicans* strains (13, 26), implicating this residue as an important amino acid for Fks1p activity and echinocandin resistance. *Candida kefyr* isolate C116, which demonstrated reduced susceptibilities to all three echinocandins, had lost this amino

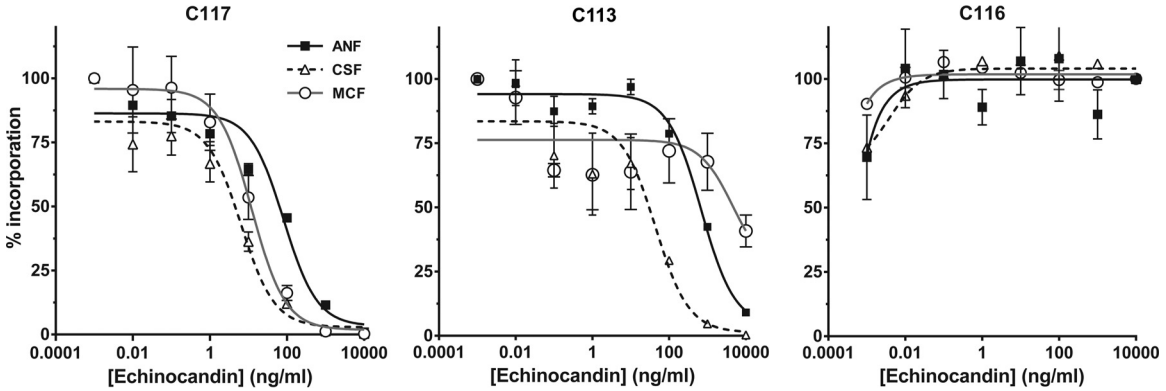

FIG 3 Echinocandin inhibition profiles of enriched GS complexes from the susceptible and resistant *C. kefyr* isolates. The *in vitro* inhibition of product-entrapped 1,3-β-D-glucan synthase (GS) complexes isolated from three *C. kefyr* strains was performed to determine the 50% inhibitory concentration (IC_{50}). Incorporation of [^{3}H]glucose into the polymerized product was measured in GS isolated from one sensitive strain (C117) and two RES strains (C113 [R647G] and C116 [F641Δ]). The GS complexes prepared from isolates C116 and C133 were insensitive to echinocandins at up to 10,000 ng/ml. Error bars represent standard errors of the means (SEM). ANF, anidulafungin; CSF, caspofungin; MCF, micafungin.

acid, with a GS enzyme complex that demonstrated reduced binding to all three drugs. This is the first report to associate loss of F641 (F641Δ) in Fks1p or its orthologous amino acid with echinocandin resistance. A homologous deletion in the *C. glabrata* Fks2p (F659Δ) also confers RES in that species (27, 32). The relative fitness of C116 has not been studied in detail, but no obvious

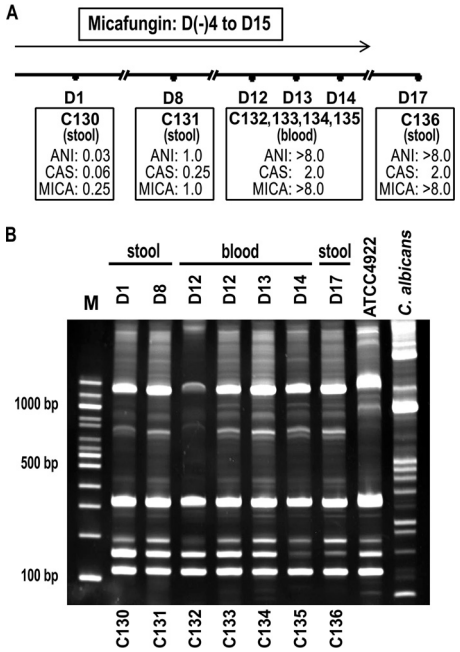

FIG 4 Genetic analysis of *C. kefyr FKS1* HS1 from a series of isolates recovered from a single patient. (A) Time line of sequential *C. kefyr* isolates. Micafungin was stopped 4 days prior to the last culture. (B) The amplicons generated by Rep-PCR with primers Ca-21 and Ca-22 were separated in a 6% TBE acrylamide gel. The day and source of culture are shown above; strain designations are shown below each lane. A study-independent *C. kefyr* (ATCC 4922), was included for comparison as was *C. albicans* (right two lanes, respectively). Isolate C132 produced the same banding pattern from independent Rep-PCRs of genomic DNA replicates. Molecular size markers are shown on the left (lane M). ANI, anidulafungin; CAS, caspofungin; MICA, micafungin.

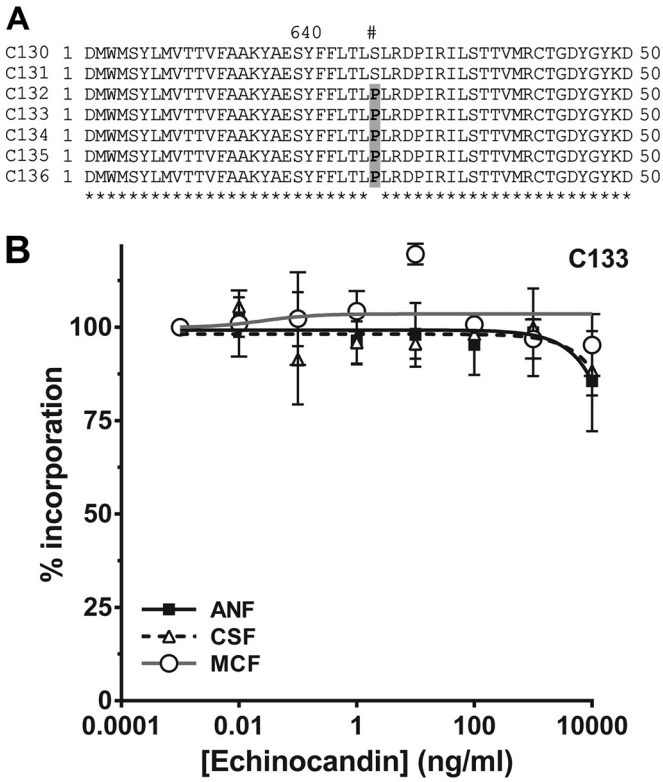

FIG 5 Molecular and biochemical analysis of the *CkFKS1* and GS complex from a series of isolates recovered from a single patient. (A) DNA sequence analysis of HS1 of isolates C130 to C136. RES isolates C132 to C136 harbor the most common amino acid substitution within HS1 leading to echinocandin resistance (S645P; shaded in gray). Amino acid residue numbers (above) are relative to the *C. albicans* Fks1p (Gsc1p/Orf19.2929p) (*Candida* Genome Database, http://www.candidagenome.org/). (B) Echinocandin inhibition assay of the enriched GS complex isolated from the representative isolate C133. The incorporation of [^{3}H]glucose into the polymerized product was measured as a function of the echinocandin concentration. The GS complex from this isolate was insensitive to all three echinocandins up to 10,000 ng/ml. Error bars represent standard errors of the means (SEM).

in vitro growth phenotype was observed. Recovery of the isolate from a patient's blood culture suggested little loss of infectivity.

The detailed study of sequential *C. kefyr* isolates recovered from one patient confirms that mucosal isolates can become resistant to all three echinocandins after prolonged administration of micafungin. Isolate C133 had particularly high MICs to all three echinocandins but displayed a lower MIC to caspofungin, repeatedly measured at 2 μg/ml (although a value above the susceptible range). This isolate harbored the most common HS1 amino acid substitution associated with RES in *C. albicans* and *C. glabrata* at amino acid 645 (S645P) (13). The extracted GS enzyme complex was relatively resistant to all three drugs, suggesting that other cellular factors impacted the apparent differential MIC.

One particularly interesting bloodstream isolate (C113) exhibited an unusual *in vitro* MIC pattern with relatively reduced susceptibility to micafungin compared to that for the other echinocandins. This isolate exhibited a novel substitution in HS1, R647G. GS inhibition studies revealed an enzyme much less susceptible to micafungin inhibition than to inhibition by either anidulafungin or caspofungin. This phenotype is similar to that observed in *C. albicans* isolates that harbor P649H (33). These data suggest that the amino acids R647 and P649 are particularly important for GS inhibition by micafungin. Definitive confirmation of this finding requires mutagenesis and reintroduction of the gene into an echinocandin-sensitive isolate.

An unexpected finding was the high level of echinocandin resistance (IC_{50} of >10,000 ng/ml) of some of the *C. kefyr* GS-bearing amino acid changes in HS1, and this correlated with MIC values of >8.0 μg/ml. This level of resistance has not been documented previously in the literature, and its significance is not yet understood. Our findings suggest that mutations in *CkFKS1* HS1 are readily generated *in vivo*, do not appear to affect strain fitness, and at times produce highly resistant echinocandin strains. Similarly, Fekkar et al. (14) reported on an RES *C. kefyr* harboring an HS1 amino acid change (F641Y) from the bloodstream of a patient with leukemia.

In conclusion, *C. kefyr* is a rare (34) but emerging cause of IC in vulnerable patient populations, such as patients with hematologic malignancies (14, 15). Our data suggest high rates of echinocandin resistance among *C. kefyr* blood isolates in AML patients with prior drug exposure, associated with specific mutations in the *CkFKS1* HS1. In addition, analysis of these isolates suggests differential echinocandin binding to the GS enzyme complex. Further study of this emerging species may provide more information on how these drugs interact with the GS enzyme complex to impart antifungal activity.

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