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Specific Substitutions in the Echinocandin Target Fks1p Account for Reduced Susceptibility of Rare Laboratory and Clinical *Candida* sp. Isolates

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An association between reduced susceptibility to echinocandins and changes in the 1,3-β-D-glucan synthase (GS) subunit Fks1p was investigated. Specific mutations in *fks1* genes from *Saccharomyces cerevisiae* and *Candida albicans* mutants are described that are necessary and sufficient for reduced susceptibility to the echinocandin drug caspofungin. One group of amino acid changes in ScFks1p, ScFks2p, and CaFks1p defines a conserved region (Phe 641 to Asp 648 of CaFks1p) in the Fks1 family of proteins. The relationship between several of these *fks1* mutations and the phenotype of reduced caspofungin susceptibility was confirmed using site-directed mutagenesis or integrative transformation. Glucan synthase activity from these mutants was less susceptible to caspofungin inhibition, and heterozygous and homozygous Ca*fks1 C. albicans* mutants could be distinguished based on the shape of inhibition curves. The *C. albicans* mutants were less susceptible to caspofungin than wild-type strains in a murine model of disseminated candidiasis. Five *Candida* isolates with reduced susceptibility to caspofungin were recovered from three patients enrolled in a clinical trial. Four *C. albicans* strains showed amino acid changes at Ser 645 of CaFks1p, while a single *Candida krusei* isolate had a deduced R1361G substitution. The clinical *C. albicans* mutants were less susceptible to caspofungin in the disseminated candidiasis model, and GS inhibition profiles and DNA sequence analyses were consistent with a homozygous *fks1* mutation. Our results indicate that substitutions in the Fks1p subunit of GS are sufficient to confer reduced susceptibility to echinocandins in *S. cerevisiae* and the pathogens *C. albicans* and *C. krusei*.

Caspofungin (CAS; L-743,872; MK-0991) is a member of the echinocandin class of antifungal compounds that noncompetitively inhibit 1,3-β-D-glucan synthase (GS), the enzyme required for formation of the essential polymer 1,3-β-D-glucan found in cell walls of most medically important fungi (9). CAS is a semisynthetic analogue of pneumocandin B_o with broad-spectrum activity against a variety of clinically important yeasts and moulds, including *Candida* and *Aspergillus* species (8, 18, 32, 33) as well as triazole-resistant strains of *Candida* (33, 35). Caspofungin has been developed as a broad-spectrum parenteral antifungal agent (7, 23) and has been approved in the United States and other countries for the treatment of a number of serious fungal infections, including invasive aspergillosis in patients who are refractory to or intolerant of other therapies, esophageal candidiasis, candidemia, and other *Candida* infections (including intra-abdominal abscesses, peritonitis, and pleural space infections). Caspofungin is also indicated for empirical therapy of suspected fungal infections in patients with persistent fever and neutropenia.

Rare clinical isolates of *Candida* with reduced in vitro susceptibility to caspofungin have been described previously (19). A correlation between in vivo failure and rising in vitro caspofungin MIC has been noted in some cases (19), although a strict correlation between MICs and clinical outcome has not been established (27). For this reason, the term "reduced susceptibility" is a more appropriate description of strains with elevated MICs than the classical term "resistant." The incidence of reduced susceptibility to caspofungin by various clinical isolates of *Candida* and *Aspergillus* species appears infrequent (39).

Previously, rare spontaneous *Candida albicans* mutants were selected in the laboratory for reduced susceptibility to L-733560, a close structural analog of CAS, and four independent mutants were characterized (22). Effective treatment of mice infected with these mutants required higher doses of L-733560 to achieve 99% reduction in kidney burden (ED_{99}) than equivalent infections with wild-type *C. albicans* (10). Susceptibility of the mutants to amphotericin B in this model of disseminated candidiasis was unaffected.

Reduced susceptibility to echinocandins has been attributed to changes in Fks1p, an essential component of the GS complex, based on genetic studies in *Saccharomyces cerevisiae* (11, 12) and *C. albicans* (10). The data further suggested that three of the *C. albicans* laboratory mutants were heterozygous for a dominant or semidominant mutation that could occur in either allele of Ca*FKS1*, while the fourth strain was believed to be a homozygous Ca*fks1* mutant.

To further understand the role of CaFks1p in echinocandin susceptibility, we have identified mutations in the coding region of Sc*FKS1* and Ca*FKS1* (also known as *GSC1*) (26), from both laboratory and rare clinical isolates, that confer reduced

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TABLE 1. Laboratory and clinical mutant strains used in this study

Strain	Genotype or origin, source, and day of therapy	Reference or source
S. cerevisiae strains		
YFK978	*MAT***a** *ade2-101 his3-Δ200 leu2- Δ1 lys2-801 trp1-Δ1 ura3-52 fks1-1 cnb1*::*LYS2 fks2-1* (pDL1)	This work
R560-1C	*MAT***a** *ade2-1 can1 his3-11,15 leu2-3,112 trp1-1 ura3-1 fks1-2*	12
MS10	*MAT***a** *fks1-3*	15
MS14	*MAT***a** *fks1-4*	15
YFK931-7B	*MAT***a** *ade2-101 his3-Δ200 leu2-Δ1 lys2-801 trp1-Δ1 ura3-52 fks1-1 cnb1*::*LYS2(pDL1)*	This work
YLIP179	*MATα ade2-1 can1 his3-11,15 leu2-3,112 trp1-1 ura3-1 fks1*::*HIS3*	11
C. albicans laboratory strains		
CA14	*Δura3*::*imm434*/*Δura3*::*imm434*	16
CA14-R1	*Δura3*::*imm434*/*Δura3*::*imm434* Ca*fks1h-1*/Ca*FKS1*	22
NR2	*Δura3*::*imm434*/*Δura3*::*imm434* Ca*fks1h-2*/Ca*FKS1b*	22
NR3	*Δura3*::*imm434*/*Δura3*::*imm434* Ca*fks1h-3*/Ca*fks1h-3*	22
NR4	*Δura3*::*imm434*/*Δura3*::*imm434* Ca*FKS1*/Ca*fks1b-4*	22
T25	*Δura3*::*imm434*/*Δura3*::*imm434* Ca*fks1h-1*/Ca*fks1b*::*URA3*	10
T28	*Δura3*::*imm434*/*Δura3*::*imm434* Ca*fks1h-1*::*URA3*/Ca*FKS1*	10
T1FOA	*Δura3*::*imm434*/*Δura3*::*imm434* Ca*fks1h*::*hisG*/Ca*FKS1b*	10
Candida clinical strains		
CLY16996 (*C. albicans*)	Patient A, blood, day 14	This work
CLY16997 (*C. albicans*)	Patient A, lung, postmortem	This work
CLY19230 (*C. albicans*)	Patient B, oral mucosa, day 27	This work
CLY19231 (*C. albicans*)	Patient B, oral mucosa, day 28	This work
CLY16038 (*C. krusei*)	Patient C, stool, day 17	This work

susceptibility to echinocandins. A method for purification of GS from *C. albicans* and *C. krusei* was developed, and the elevated caspofungin 50% inhibitory concentration (IC_{50}) values suggest that target-site modifications are responsible for changes in whole-cell susceptibility. Finally, the identification of genetically related isolates from the same patient, with different mutations in *FKS1*, suggests that reduced susceptibility can evolve in the patient.

MATERIALS AND METHODS

Strains and compounds. The laboratory and clinical mutants used in this study are shown in Table 1. *S. cerevisiae* strains MS10 and MS14 (15) were provided by M. el-Sherbeini (Merck, Rahway, NJ). Laboratory *C. albicans* mutants (10) were from the Merck culture collection (Rahway, NJ); for in vivo experiments, the Ura^- auxotrophs were transformed to Ura^+ with plasmid pJAM15 (10). CAI4 (16) was kindly provided by William Fonzi (Georgetown U). Clinical isolates (n = 37) included *C. albicans* (n = 9), *C. krusei* (n = 3), *C. guilliermondii* (n = 2), *C. glabrata* (n = 3), *C. tropicalis* (n = 5), and *C. parapsilosis* (n = 15). *C. albicans* strains GU5, B5, and FO1 for multilocus sequence typing (MLST) profiling were provided by Joachim Morschhäuser (Universität Würzburg, Würzburg, Germany) and Frank Odds (University of Aberdeen, Aberdeen, UK). CAS and L-733560 (6) were obtained from Merck (Rahway, NJ) and were dissolved in sterile distilled water unless indicated otherwise. These compounds are closely related diamine-substituted analogs of pneumocandin B_0 with equivalent in vitro antifungal activity (4, 5).

MIC determinations. Susceptibility to caspofungin or L-733560 was measured in liquid broth microdilution assays. Cultures were grown in either RPMI 1640 medium supplemented with 0.165 M MOPS (morpholinepropanesulfonic acid) (pH 7.0), AM3 (30) or SD (12) medium, or YPD broth (10), as indicated. For all clinical *Candida* isolates, the method outlined in CLSI (formerly NCCLS) document M27-A2 was used to determine caspofungin MICs (29). The susceptibility of *C. albicans* strains CAI4-R1, NR2, NR3, NR4, and T25 (Table 1) to caspofungin was also evaluated according to protocol M27-A2, except that absorbance was read in a spectrophotometer and the MIC was defined as the lowest caspofungin concentration that reduced the optical density at 600 nm (OD_{600}) to that of a media blank. For *S. cerevisiae* strains and *C. albicans* transformants, susceptibility to L-733560 was determined by absorbance, measured after 24 h of growth at 30°C. Details for these assays (media used, MIC definitions) are given in the table legends. The working definition for reduced susceptibility in this study refers to a property of rare strains, both laboratory and clinical, that require at least fivefold more drug to prevent growth in phenotypic and in vivo assays relative to a parental wild-type for lab strains or >95 percent of clinical isolates in a given genus for clinical strains.

***S. cerevisiae fks* alleles.** The multicopy, URA3-based plasmid pJAM54 (11), carrying a full-length Sc*FKS1* gene (GenBank accession no. U12893), was used for gap repair cloning (28) and DNA sequence analysis of rescued Sc*fks1* gene fragments from spontaneous echinocandin-resistant *S. cerevisiae* mutants R560-1C, MS10, and MS14 (12, 15). The spontaneous Sc*fks2* mutant YFK978 (Table 1) bearing plasmid pDL1 (*ARS1 CEN1 URA3 SUP11 CNB1*) was selected for growth on agar plates containing L-733560. A portion of the *fks2-1* allele from strain YFK0978 was amplified by PCR, cloned, and sequenced using standard procedures.

Isolation of Ca*Fks1h-1* and introduction into T1FOA. Ca*fks1h-1* was isolated by a targeted integration/excision strategy. pGSC2, a plasmid containing the 3′ end of Ca*FKS1*, was constructed by subcloning a 3.1-kb XbaI-HindIII Ca*FKS1* fragment from pJAD2 (10) and a 1.3-kb XbaI-ScaI *URA3* fragment of pJAM11 (40) into pGEM3zf (Promega). Ura^+ transformants of CAI4-R1 were obtained with pGSC2 linearized with Csp451 by use of a transformation procedure previously described for *S. cerevisiae* (14). Clones with pGSC2 integrated into the allele of Ca*FKS1* responsible for reduced susceptibility (designated Ca*fks1h-1*) were identified by Southern blot analysis, taking advantage of a HindIII restriction site polymorphism between alleles (10). The integrated plasmid and adjacent DNA containing Ca*fks1h-1* were recovered by digestion of total genomic DNA with HindIII, followed by ligation and transformation of *Escherichia coli* with selection for ampicillin-resistant colonies. This plasmid (pGSC3) contained all of Ca*fks1h-1* with the exception of the 5′ noncoding sequence and DNA encoding the first 251 amino acids. In addition, pGSC3 had a duplication of the 3.1-kb 3′ end of the Ca*FKS1* sequence from pGSC2 and the genome. Plasmid pGSC8 was constructed through a series of subcloning steps. It contained the 6.0-kb HindIII-NruI Ca*Fks1h-1* fragment of pGSC3 fused in frame to a 2.5-kb Ca*FKS1* HindIII fragment (containing wild-type noncoding sequence and sequence corresponding to the first 251 amino acids) and the 1.2-kb URA3 fragment (Fig. 1). Strain T1FOA (Ca*fks1h*::*hisG*/Ca*FKS1b*) (10) was transformed with SpeI-digested pGSC8, and Ura^+ transformants were selected. The Ura^+ transformants were subsequently tested for growth on Ura^- medium containing 0.8 μg/ml L-733560. To determine whether pGSC8 had integrated at the Ca*FKS1*

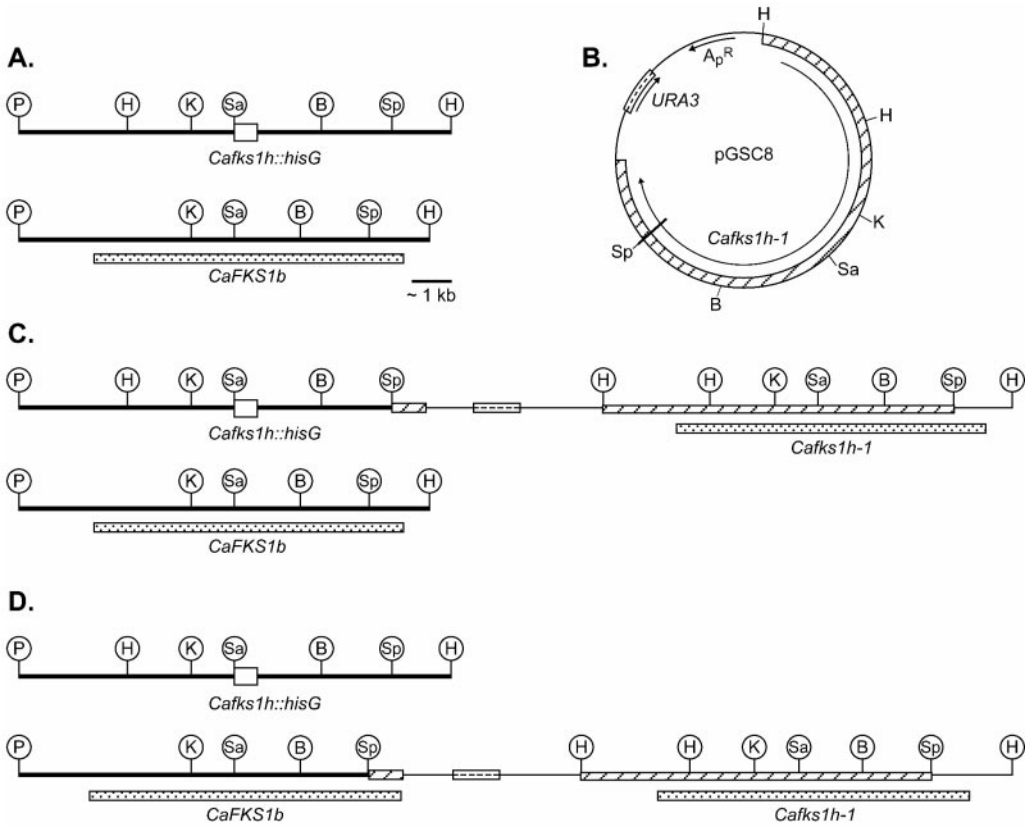

FIG. 1. Integration of plasmid pGSC8 at Ca*FKS1*. (A) Schematic map of the Ca*fks1* locus of strain T1FOA, showing the Ca*fks1h*::*hisG* and Ca*FKS1b* alleles. Abbreviations for restriction enzyme cleavage sites are as follows: P, PvuII; H, HindIII; K, KpnI; Sa, SalI; B, BamHI; Sp, SpeI. The K, Sa, and B sites are included for reference only and should not be considered unique within the regions shown. The open box represents the *hisG* sequence remaining after the Ura blaster method (16) was used to construct a Ura$^-$ strain with a deletion of Ca*FKS1*. Rectangles with stippling below the alleles indicate regions encoding either CaFks1p or Cafks1p. (B). Map of plasmid pGSC8. The pBR322 vector sequences (thin line), the 1.2-kb *URA3* gene (stippled line), and DNA at the Ca*fks1h-1* locus (hatched line) are indicated. Arrows within the circle illustrate the direction of transcription of the *URA3*, Ca*FKS1*, and *E. coli bla* genes. The plasmid was linearized by digestion with SpeI (thick line intersecting the circle) prior to transformation. (C and D) Chromosomal maps following pGSC8 integration at Ca*FKS1*. The maps depict pGSC8 integration at the Ca*fks1h*::*hisG* allele (C) or the Ca*FKS1b* allele (D).

locus, Southern blot analysis was performed as described previously (10). The allele reconstructed by integration of pGSC8 contains 90% of the coding sequence derived from Ca*fks1h-1*; the 251 N-terminal and 363 C-terminal amino acids were derived from wild-type sequence.

DNA sequence analysis of *FKS1* from laboratory mutants and clinical isolates. A region of the Ca*FKS1* open reading frame was chosen for sequence analysis based on the position of Sc*fks* mutations that conferred reduced echinocandin susceptibility to *S. cerevisiae* strains R560-1C, MS10, and YFK978. Fragments of Ca*FKS1* (ca. 450 bp) were amplified from genomic DNA from strains CAI4-R1, NR2, NR3, and NR4. The sense and antisense primers used for PCR, based on the Ca*FKS1* (*GSC1*) sequence (GenBank accession no. D88815), were 5′-GAA ATCGGCATATGCTGTGTC-3′ and 5′-AATGAACGACCAATGGAGAAG-3′, respectively. PCR products were cloned into pCR2.1 (Invitrogen), and the DNA sequence was determined. For clinical *Candida* isolates, the entire Ca*FKS1* open reading frame was sequenced. A 2.4-kb fragment of *FKS1* from *C. krusei* was amplified using primers 5′-TACTGCATCGTTTGCTCCTCT and 5′-CGA GCACCACCAATGGAAAC and then sequenced (GenBank accession no. DQ017894).

Site-directed mutagenesis. Fragments of the *S. cerevisiae* Sc*FKS1* gene were used for unique site elimination mutagenesis (Stratagene, La Jolla, CA). In addition to three Sc*fks1* mutations, the Sc*fks2-1* and Ca*fks1h-1* mutations were introduced at the equivalent positions in Sc*FKS1*. The wild-type region of Sc*FKS1* in a Yeplac181-derived vector (17) was replaced with mutated fragments, and each plasmid was introduced into YLIP179, an *fks1*Δ null strain (11), for characterization. Whole-cell susceptibility and GS enzyme inhibition by L-733560 were measured as described previously (10) and compared to values determined for strains bearing a plasmid-encoded, wild-type Sc*FKS1* gene.

Isolation and assay of GS from *Candida*. Each *Candida* isolate was grown with vigorous shaking at 30°C to early stationary phase in YPD medium, and cells were collected by centrifugation. Washed pellets were disrupted with 0.5-mm glass beads in 50 mM HEPES (pH 7.4)–10% glycerol–1 mM EDTA–1 mM phenylmethylsulfonyl fluoride–1 mM dithiothreitol. Membranes were isolated by sedimentation at 100,000 × *g* and washed twice. Extraction was performed at a protein concentration of 5 mg/ml (Bradford assay; Bio-Rad) in extraction buffer [50 mM $NaPO_4$ pH 7.5, 0.1 M KCl, 0.1 M Na citrate, 20% glycerol, 5 μM GTP-γ-S (guanosine 5′-O-(3-thiotriphosphate)), 1 mM dithiothreitol, 1 mM phenylmethylsulfonyl fluoride, 3 μg/ml pepstatin] with 0.25% (vol/vol) W1 (polyoxyethylene ether) by gentle mixing at 4°C for 60 min, followed by centrifugation at 100,000 × *g* for 60 min. Enzymatic activity was partially purified by a modified product entrapment procedure (20). First, crude membranes were diluted fivefold in trapping buffer (50 mM HEPES [pH 7.5], 10 mM KF, 1 mM EDTA, 2 mg/ml bovine serum albumin) containing 2.5 mM UDPG (uridine 5′-diphosphoglucose) and 20 μM GTP-γ-S. After incubation at 25°C for 60 min, glucan was harvested by low-speed centrifugation (3,000 × *g* for 10 min). Washing and subsequent extraction were performed essentially as described by Kondoh et al. (21).

The sensitivity to caspofungin was measured in a polymerization assay in a 96-well format. Each well contained [^{3}H]UDPG at 0.5 mM (6,000 to 8,000 dpm/nmol), 50 mM HEPES (pH 7.5), 10% (wt/vol) glycerol, 1.5 mg/ml bovine serum albumin, 25 mM KF, 1 mM EDTA, 25 μM GTP-γ-S, and sufficient

enzyme to yield 3 to 6 nmol of radiolabeled product following 60 min of incubation at 22°C, in a final volume of 100 μl. Serial dilutions of caspofungin dissolved in 100% dimethyl sulfoxide were added (1 μl/well) at the start of incubation. Reactions were stopped by the addition of 100 μl of 20% trichloroacetic acid. Plates were chilled for a minimum of 10 min, and precipitated glucan was collected by filtration on GF/C plates (Packard Unifilter-96) and washed with five cycles of water (about 1 ml/well/cycle) using a Packard Filtermate Harvester. Scintillation fluid (40 μl/well; Packard Ultima Gold-XR) was added, and sealed plates were counted in a Wallac Beta counter in top-counting mode at an efficiency of approximately 40%. Results were analyzed (Prism Software, Irvine, CA) using the sigmoidal response (variable slope) curve fitting algorithm.

Molecular genotyping by multilocus sequence typing (MLST). Multilocus sequence typing based on six housekeeping genes was performed on the clinical isolates of *C. albicans*, as described by Robles et al. (36). An unweighted pair group method dendrogram based on the pair-wise differences in the allelic profiles of the six housekeeping genes was made using web-based tree design software (http://calbicans.mlst.net/sql/uniquetree.asp?).

Murine model of disseminated candidiasis. For *C. albicans* strains, C′5-deficient DBA/2J (Jackson Laboratories, Bar Harbor, ME) or DBA/2N (Taconic, Germantown, NY) female mice weighing 19 to 21 g were used. For *C. krusei*, CD-1 mice (Charles River) weighing 22 to 27 g were immunosuppressed with cyclophosphamide as described previously (2). A disseminated infection was established with each *Candida* isolate, using approximately 1 times the 50% lethal dose as the inoculum. Therapy was initiated 15 to 30 min after challenge. Mice were treated with vehicle or caspofungin (titrated in twofold increments from 0.03 to 20 mg/kg of body weight/dose) administered intraperitoneally once a day for a total of 4 days. On day 4 postinfection, kidneys from euthanized mice (5 per group, unless specified) were evaluated for *Candida* CFU as described previously (2). Caspofungin efficacy measurements were based on differences in CFU/g kidney using Student's *t* test (two-tailed, unpaired). Inverse regression was subsequently used to estimate ED_{90} values, defined as the dose (mg/kg) that reduced the number of CFU per organ by 90% compared to sham-treated control mouse results.

Clinical isolates. Protocol 026 was a phase III double-blind, randomized, comparative registration trial of caspofungin versus liposomal amphotericin B as empirical therapy for suspected fungal infections in adults with persistent fever and neutropenia (41). A survey of *Candida* strains recovered from patients enrolled in this study identified five isolates (from three patients) with caspofungin MICs ≥ 4 μg/ml (Table 1). For one patient (patient A) with a fatal breakthrough of acute disseminated candidiasis (onset day 14), *C. albicans* isolates with a caspofungin MIC of >8 μg/ml were recovered from blood on day 14 of therapy and from postmortem culture of lung. Additional *C. albicans* strains, with caspofungin MICs = 0.5 μg/ml, were obtained from the oral mucosa and liver of patient A on day 0 and postmortem, respectively. The second patient (patient B) received study therapy for 6 weeks and was diagnosed with breakthrough chronic disseminated candidiasis (onset at 5[1/2] weeks on study). Two *C. albicans* isolates with a caspofungin MIC of 0.5 μg/ml were recovered from the urine of patient B on day 4 of therapy, and two *C. albicans* isolates with a caspofungin MIC of 4 μg/ml were obtained from oral mucosa on days 27 and 28 of therapy (Table 1). The fifth isolate, with a caspofungin MIC of 32 μg/ml, was a *C. krusei* strain recovered from stool on day 17 of therapy from a third patient (patient C) with a fatal breakthrough *C. krusei* fungemia.

Fluctuation test. The frequency of spontaneous mutations conferring decreased caspofungin susceptibility to clinical isolate *C. albicans* CLY18195 (patient A, oral mucosa, day 0) was measured essentially as described previously (22, 24). Cells in 20 replicate 1-ml cultures were grown at 30°C in YPD medium from a seed density of 25 cells/ml to a final density of approximately 2×10^7 cells/ml. The cell density was determined by microscopy with a hemocytometer. Spontaneous mutants were detected on YPD agar plates containing 1 μg/ml caspofungin, which was eightfold higher than the amount of drug (0.125 μg/ml) needed to prevent growth under these conditions. This caspofungin concentration did not prevent growth of the spontaneous *C. albicans* laboratory C*afks1* mutants CAI4-R1 and NR3. The rate of spontaneous mutation was determined using the following formula: $a = (-\ln p_0)/N$, where a is the rate of spontaneous mutation per cell per generation, p_0 is the fraction of negative plates, and N is the final culture cell density measured by hemocytometer counting.

RESULTS

***S. cerevisiae* Sc*fks* mutations.** Four previously described *S. cerevisiae* mutants with reduced susceptibility to the echinocandin L-733560 (9, 12, 15) (Table 2) were evaluated for mutations in the Sc*FKS* coding sequence. Gap repair cloning (28) was used to identify a region of the Sc*fks1* gene from strains R560-1C and MS10 associated with reduced susceptibility. DNA sequence analysis of the recovered Sc*fks1* gene fragments revealed a deduced F639I substitution in the mutant ScFks1 protein of strain R560-1C and a replacement of Asp646 with Tyr in mutant MS10. Mutations in this region of Sc*fks* were also identified in a gene fragment cloned from the Sc*fks2* mutant YFK978. The Sc*FKS2* gene encodes an alternate subunit of the GS complex with significant homology (87% identity) to ScFks1p and a distinct expression pattern (9). The Sc*fks2* mutant had a deduced I660K substitution, which aligned with Val 641 of ScFks1p (Fig. 2). The *S. cerevisiae* mutant strain MS14 (15) did not have a mutation in this region of Sc*FKS1*, but a deduced Arg-to-Ser substitution at amino acid

TABLE 2. Echinocandin susceptibility of *S. cerevisiae* Sc*FKS* wild-type strains and mutants derived from them

Strain	Sc*FKS1* allele	Susceptibility to L-733560	
		Whole-cell MIC[a] (μg/ml)	GS IC_{50}[b] (μM)
W303-1A	Wild-type Sc*FKS1*	0.4	0.9
Sc*fks1*Δ (pFKS1)	Plasmid-encoded Sc*FKS1*	0.4	0.4
R560-1C	Sc*fks1-2*	16	25
Sc*fks1*Δ (pfks1-2)	F639I	8	20
X2180-1A	Wild-type Sc*FKS1*	0.6	0.9
MS10	Sc*fks1-3*	6	20
Sc*fks1*Δ (pfks1-3)	D646Y	4	4
MS14	Sc*fks1-4*	8	5
Sc*fks1*Δ (pfks1-4)	R1357S	10	3
YFK931-7B	Sc*fks1-1*[c]	0.1	0.3
YFK978	Sc*fks1-1* Sc*fks2-1*	3	45
Sc*fks1*Δ (pfks2-1)	V641K	8	35

[a] Strains were grown in either SD (wild-type and *fks1* mutants), SD-leucine (pfks1 transformants) or YPD (*fks1-1* mutants). Cells from mid-log phase cultures were diluted to $OD_{600} < 0.01$ and used as inocula. Absorbance was read after 24 h at 30°C; the MIC was defined as the lowest drug concentration that reduced optical density to ≤ 10% of growth in drug-free control wells.

[b] Inhibition of crude membrane-associated GS activity was measured as described in Materials and Methods. L-733560 was titrated from 0.02 to 50 μM in the reactions.

[c] Sc*fks1-1* is a loss-of-function allele (11).

ScFks1p:	Phe_{639}	Leu	Val	Leu	Ser	Leu	Arg	Asp_{646}
ScFks1-2p:	Ile_{639}	Leu	Val	Leu	Ser	Leu	Arg	Asp_{646}
ScFks1-3p:	Phe_{639}	Leu	Val	Leu	Ser	Leu	Arg	Tyr_{646}
ScFks2p:	Phe_{658}	Leu	Ile	Leu	Ser	Leu	Arg	Asp_{645}
ScFks2-1p:	Phe_{658}	Leu	Lys	Leu	Ser	Leu	Arg	Asp_{645}
CaFks1p:	Phe_{641}	Leu	Thr	Leu	Ser	Leu	Arg	Asp_{648}
CaFks1h-1p:	Phe_{641}	Leu	Thr	Leu	Pro	Leu	Arg	Asp_{648}
CaFks1h-3p:	Phe_{641}	Leu	Thr	Leu	Tyr	Leu	Arg	Asp_{648}

FIG. 2. Alignment of Fks protein sequences important for echinocandin susceptibility in *S. cerevisiae* and *C. albicans*. The Fks proteins from wild-type *S. cerevisiae* (ScFks1p and ScFks2p; GenBank accession numbers U12893 and U16783, respectively) and *S. cerevisiae* mutants R560-1C (ScFks1-2p), MS10 (ScFks1-3p), and YFK978 (ScFks2-1p), as well as wild-type *C. albicans* (CaFks1p; accession number D88815) and *C. albicans* mutants CAI4-R1 (CaFks1h-1p) and NR3 (CaFks1h-3p), were aligned. Subscripts for the first and last residue of each entry identify amino acid positions in the Fksp sequence.

TABLE 3. Properties of *C. albicans* laboratory strains with reduced caspofungin susceptibility

Strain	Fks1p mutation	MIC[a] (μg/ml) in:		Glucan synthesis IC_{50} (ng/ml)	Mouse ED_{90} (mg/kg)
		RPMI	AM3		
CAI4	S645/S645	0.12	<0.06	0.91	0.002
CAI4-R1	S645/P645	>32	>2	0.5 and 100	0.14
NR2	S645/P645	>32	>2	ND[b]	0.09
NR4	P645/S645	>32	1	0.5 and 100	0.07
NR3	Y645/Y645	>32	>2	2500	3.20
T25	Δ[c]/P645	>32	>2	133	3.39

[a] Susceptibility testing was performed per CLSI protocol M27A2, except MIC values were defined as the lowest caspofungin concentration to reduce the OD_{600} to that of a media blank.
[b] Not determined.
[c] Δ indicates a disrupted Ca*fsk1* allele.

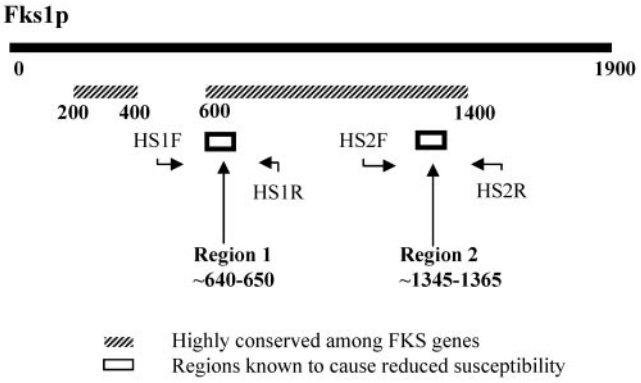

FIG. 3. Schematic diagram of Ca*Fks1* loci associated with reduced susceptibility to caspofungin. The locations of regions linked to reduced caspofungin susceptibility are shown superimposed on a linear profile of CaFks1p (numbers refer to amino acid positions). The gray bar depicts highly conserved regions of the Fks protein, and arrows provide the approximate hybridization positions of primer sets (HS1F, etc.) used to amplify the two regions associated with reduced susceptibility.

1357 of ScFks1p was identified. The relationship between the Sc*fks* mutations and reduced echinocandin susceptibility was evaluated in whole cells and in assays measuring inhibition of microsomal GS activity. Plasmid copies of the wild-type Sc*FKS1* gene, or Sc*fks1* mutant alleles created by site-directed mutagenesis, were introduced into a strain with a disruption (Δ) at the chromosomal Sc*fks1* locus. The echinocandin susceptibility of each engineered mutant was consistent with that of the original mutant, both in whole cells and in microsomal GS activity (Table 2). The sites of the Sc*fks1* mutations provided a basis for analyzing the *fks1* genes from *Candida* isolates with reduced susceptibility to echinocandins.

Amino acid 645 of CaFks1p is altered in *C. albicans* laboratory *fks1* mutants. Four rare spontaneous *C. albicans* laboratory mutants (CAI4-R1, NR2, NR3, and NR4) were shown previously to be less susceptible to L-733560 than their wild-type parent (22). In this study, there was no difference among the mutants when caspofungin MICs were determined in either RPMI-1640 or AM3 medium (Table 3). To identify Ca*fks1* mutations linked to reduced susceptibility, a fragment of the Ca*fks1* locus from each mutant was cloned and sequenced. This fragment encompasses region 1 of Ca*fks1*, which aligns with codons for all but one of the Sc*fks1* mutations defined above (Fig. 3). The codon for Ser 645 of Cafks1p was altered in all four *C. albicans* laboratory mutants. For strains CAI4-R1, NR2, and NR4, we identified a S645P substitution, while strain NR3 had a S645Y substitution. Sequence analysis of multiple Ca*fks1*-derived clones from each mutant revealed a mixture of mutated and wild-type alleles for strains CAI4-R1 (4 mutated alleles of 9 sequenced), NR2 (4/10), and NR4 (6/14) but not for strain NR3 (10 mutated alleles of 10 sequenced).

We sought to confirm the association between these Ca*fks1* mutations and the phenotype of reduced echinocandin susceptibility. *C. albicans* strains that closely resembled CAI4-R1, i.e., strains with one wild-type allele (Ca*FKS1b*) and one mutant allele (Ca*fks1h-1*) encoding the S645P amino acid substitution, were constructed. After isolating the major portion of the Ca*fks1h-1* allele associated with reduced susceptibility from strain CAI4-RI by targeted integration/excision, a plasmid containing a composite Ca*FKS1* gene (pGSC8) was transformed into T1FOA, a strain heterozygous for a disruption of Ca*FKS1* (Fig. 1). To specifically target pGSC8 to either the Ca*FKS1* or Ca*fks1* allele, it was linearized with SpeI. Integration of pGSC8 into either Ca*fks1h*::*hisG* (Fig. 1C) or Ca*FKS1b* (Fig. 1D) of T1FOA yielded Ura^+ transformants with one functional wild-type and one functional mutated gene. All of the transformants with the plasmid integrated at either Ca*FKS1b* (transformant 8) or C*afks1h*::*hisG* (transformants 1, 2, and 3) were less susceptible to L-733560 than the parental strain T1FOA transformed with a control Ura^+ plasmid (Table 4). The two transformants in which pGSC8 was not integrated at the Ca*FKS1* locus (transformants 4 and 5) were as susceptible as T1FOA (pJAM11). These results support the notion that mutations in Ca*FKS1* confer reduced susceptibility to echinocandins.

The serine residue that is altered in these mutant Cafks1 proteins is conserved between Fks1p of *C. albicans* (S645) and *S. cerevisiae* (S643; Fig. 2). It was of interest to determine whether this single amino acid change was sufficient to alter echinocandin susceptibility. A plasmid-encoded allele of Sc*FKS1* with the Ca*fks1h-1* mutation introduced at the equivalent codon (Ser 643) was created by site-directed mutagenesis and introduced into a strain with a chromosomal disruption of

TABLE 4. Echinocandin susceptibility of independent pGSC8 transformants of TIFOA

Transformant designation	Integration site	MIC[a]
1	Ca*fks1h*::*hisG*	2
2	Ca*fks1h*::*hisG*	2
3	Ca*fks1h*::*hisG*	2
8	Ca*FKS1b*	2
4	Unknown[b]	<0003
5	Unknown	<0.003
pJAM11[c] in T1FOA	*URA3*[d]	<0.003
pJAM11 in CAI4-R1	*URA3*	4

[a] The MIC for L-733560 was defined as the lowest drug concentration that reduced the OD_{600} to that of media. Values are the average of 3 independent experiments in YPD broth.
[b] Not integrated at Ca*FKS1b* or Ca*fks1h*::*hisG*.
[c] Ura* control plasmid.
[d] Site of integration unproven.

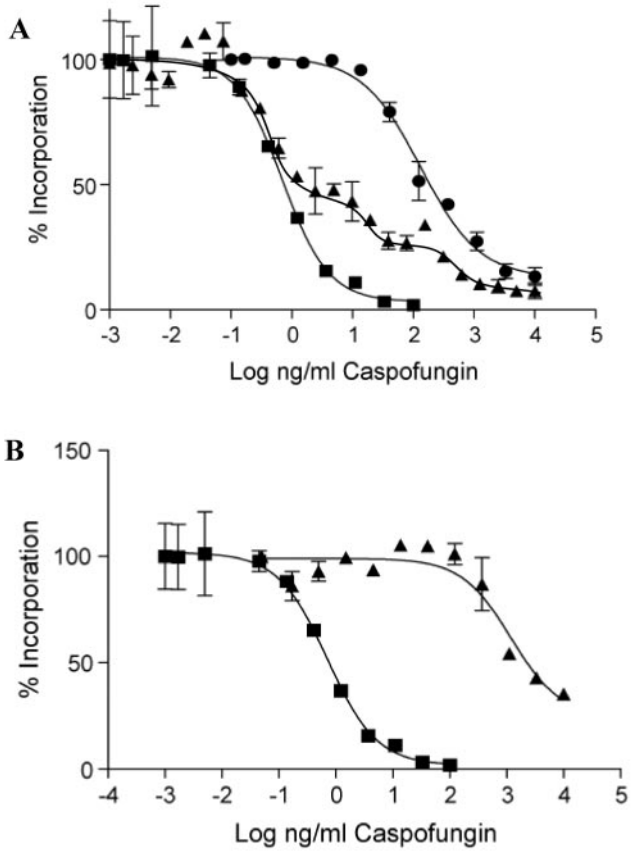

FIG. 4. Caspofungin inhibition profiles of enriched GS complexes from wild-type and mutant *C. albicans* strains. Relative GS activity was assessed by the incorporation of [^{3}H]glucose into radiolabeled product. (A) Caspofungin titration curves for the *C. albicans* parental strain CAI4 (squares), heterozygous S645P/WT strain NR4 (triangles), and homozygotic pseudo-haploid S645P/null strain T25 (circles). Percent incorporation values for GS from strain T25 did not fit a simple sigmoidal curve. (B) Caspofungin titration curves for the parental strain CAI4 (squares) and the homozygous S645Y/S645Y strain NR3 (triangles).

Sc*FKS1*. As expected, the S643P substitution in ScFks1p resulted in reduced susceptibility to L-733560, with higher MICs (32 μg/ml for the mutant versus 0.25 μg/ml for the wild type) and higher microsomal GS IC_{50} values (36 μg/ml for the mutant versus 0.48 μg/ml for the wild type).

Ser645 substitutions alter the GS inhibition profile. We were unable to distinguish between the heterozygous (CAI4-R1, NR2, NR4) and homozygous (NR3) laboratory Ca*fks1* mutants based on whole-cell susceptibility to caspofungin (Table 3). Previous work with crude GS derived from these strains suggested that the shape of inhibition curves across an L-733560 titration reflected the differences at the Ca*fks1* locus (10). To better understand the relationship between Ca*FKS1* mutations and GS susceptibility to caspofungin, drug was titrated against product-entrapped enzyme from each laboratory mutant. The enzyme inhibition profiles for caspofungin determined using product-entrapped GS preparations from the wild-type strain CAI4, heterozygous mutant strain NR4 (S645P/WT), and a strain containing a single mutant allele at the Ca*FKS1* locus, [T25; (S645P/null)], are shown in Fig. 4A. Titration curves for strains CAI4 and T25 have a single inflection point, with caspofungin IC_{50} values of 0.91 and 133 ng/ml, respectively (Table 3). The inhibition curve for the Ca*FKS1* heterozygous strain NR4 is biphasic, which most likely reflects inhibition of wild-type GS activity at low caspofungin concentrations and inhibition of S645P-containing GS at higher concentrations. Product-entrapped GS prepared from the heterozygous CAI4-R1 strain also has a biphasic caspofungin inhibition curve and two distinct IC_{50} values (Table 3). Caspofungin inhibition of GS enzyme activity from strain NR3 (S645Y/S645Y) and its parental strain CAI4 is shown in Fig. 4B. The curve for each enzyme has a single inflection point, and the GS IC_{50} value of 2,500 ng/ml for strain NR3 is more than 3 orders of magnitude higher than the value for wild-type GS (Table 3).

In vivo caspofungin susceptibility of Ca*fks1* mutants. A murine model of disseminated candidiasis was used to assess the efficacy of caspofungin against a Ura^+ derivative of each laboratory Ca*fks1* mutant. Mice were infected, treated by intraperitoneal injection once a day for 4 days beginning 15 to 30 min after infection, and assessed for fungal burden in kidneys on day 4 postinfection (24 h after the last therapy dose). ED_{90} values for the wild-type strain (0.002 mg/kg of body weight/day) and each of the Ca*fks1* mutants are shown in Table 3. Higher doses of caspofungin were required to achieve 90% reduction in kidney burden for each of the mutant strains tested. The strains containing only Ca*fks1* alleles (NR3 and T25) have ED_{90} values considerably higher than those of the heterozygous mutants CAI4-R1 and NR4.

***FKS1* mutations in clinical isolates.** Five isolates from patients enrolled in a caspofungin clinical trial (protocol 026) were identified for further evaluation based on elevated caspofungin MICs (≥4 μg/ml in RPMI 1640 medium). We amplified and sequenced from each strain the 2.6-kb fragment of Ca*FKS1* containing the targeted regions identified in laboratory Sc*fks1* and Ca*fks1* mutants (Fig. 3). As shown in Table 5, three *C. albicans* isolates (CLY16996, CLY19230, CLY19231) contain a S645F substitution in CaFks1p, while a fourth *C. albicans* isolate (CLY16997) contains the same S645P mutation that was identified in several *C. albicans* laboratory mutants. Subsequent full-length sequencing of each Ca*fks1* gene did not reveal additional mutations conferring amino acids substitutions. For all four clinical isolates, the chromatogram generated from DNA sequence analysis of genomic DNA revealed a single nucleotide at the site of the mutation, which is consistent with a homozygous change at the Ca*fks1* locus (data not shown). Evaluation of five epidemiologically distinct caspofungin-susceptible (MICs = 0.5 μg/ml) *C. albicans* isolates from the clinical study revealed no nucleotide changes among these strains in the same 2.6-kb portion of Ca*FKS1*. In addition, more than 50 other *C. albicans* clinical isolates with wild-type susceptibility to caspofungin failed to show mutations in the target regions of Ca*FKS1* (data not shown). The fifth clinical isolate with reduced caspofungin susceptibility (MIC = 32 μg/ml) was a *C. krusei* strain (CLY16038). The *fks1* gene fragment from strain CLY16038 had a predicted R1361G substitution, which aligns with the position of the R1357S Fks1p substitution identified in *S. cerevisiae* mutant MS14 (Table 2) (region 2 in Fig. 3). DNA sequence analysis suggested that the

TABLE 5. Properties of clinical *Candida* isolates from protocol 026

Patient	Species and CLY no.	Amino acid change in Fkslp	MIC (μg/ml) in:		Glucan synthesis IC_{50} (ng/ml)	Mouse LD_{50} (CFU/mouse $\times 10^{-3}$)	Mouse ED_{90} (mg/kg/day)
			RPM1	AM3			
A	*C. albicans* CLY16996	S645F	>8	2	1,997	440	1.09
	C. albicans CLY16997	S645P	>8	4	162	190	9.98
	C. albicans CLY16998	None	0.5	0.06	0.56	110	<0.06
	C. albicans CLY18195	None	0.25	0.06	0.91	600	0.01
B	*C. albicans* CLY19228	None	0.5	0.06	0.40	42	0.005
	C. albicans CLY19229	None	0.5	0.06	0.40	54	0.002
	C. albicans CLY19230	S645F	4	2	420	166	0.76
	C. albicans CLY19231	S645F	4	2	456	170	0.73
C	*C. krusei* CLY16038	R1361G	32	16	795	>8,200	NC[a]

[a] Not calculated due to poor virulence of isolate.

nucleotide change was present in both alleles at the *C. krusei FKS1* locus. A caspofungin-susceptible *C. krusei* isolate from the same clinical study, as well as 10 other susceptible *C. krusei* clinical isolates, had *FKS1* genes encoding Arg at amino acid 1361 (data not shown).

We evaluated the caspofungin susceptibility of purified GS from each of the clinical isolates and assessed the efficacy of caspofungin against them in a murine model of disseminated candidiasis. The GS IC_{50} values for caspofungin were significantly higher (180- to 2,200-fold) among all five mutant clinical isolates than the IC_{50} value for wild-type enzyme (Table 5), and the titration curves had a single inflection point (data not shown). In infected mice, caspofungin ED_{90} values for the *C. albicans* mutants fell into two groups: the S645F CaFks1p mutants (strains CLY16996, CLY19230, and CLY19231) had values ranging from 0.73 to 1.09 mg/kg/day, while the S645P mutant (strain CLY16997) had an ED_{90} of 9.98 mg/kg/day. In contrast, the ED_{90} values for Ca*FKS1* wild-type *C. albicans* strains ranged from 0.002 to <0.06 mg/kg/day. We were unable to establish a reproducible infection with *C. krusei* strain CLY16038.

***FKS1* alleles from *C. albicans* strains isolated from the same patient.** Multilocus sequence typing (MLST) analysis was used to determine the genetic relatedness of strains isolated from single patients. *C. albicans* isolates CLY16996 and CLY16997, with Ca*FKS1* mutations encoding S645F and S645P substitutions, respectively, were recovered from the blood and lung of the same patient (patient A, protocol 026). Two additional isolates (CLY16698 and CLY18195) from the same patient were susceptible to caspofungin and did not contain mutations

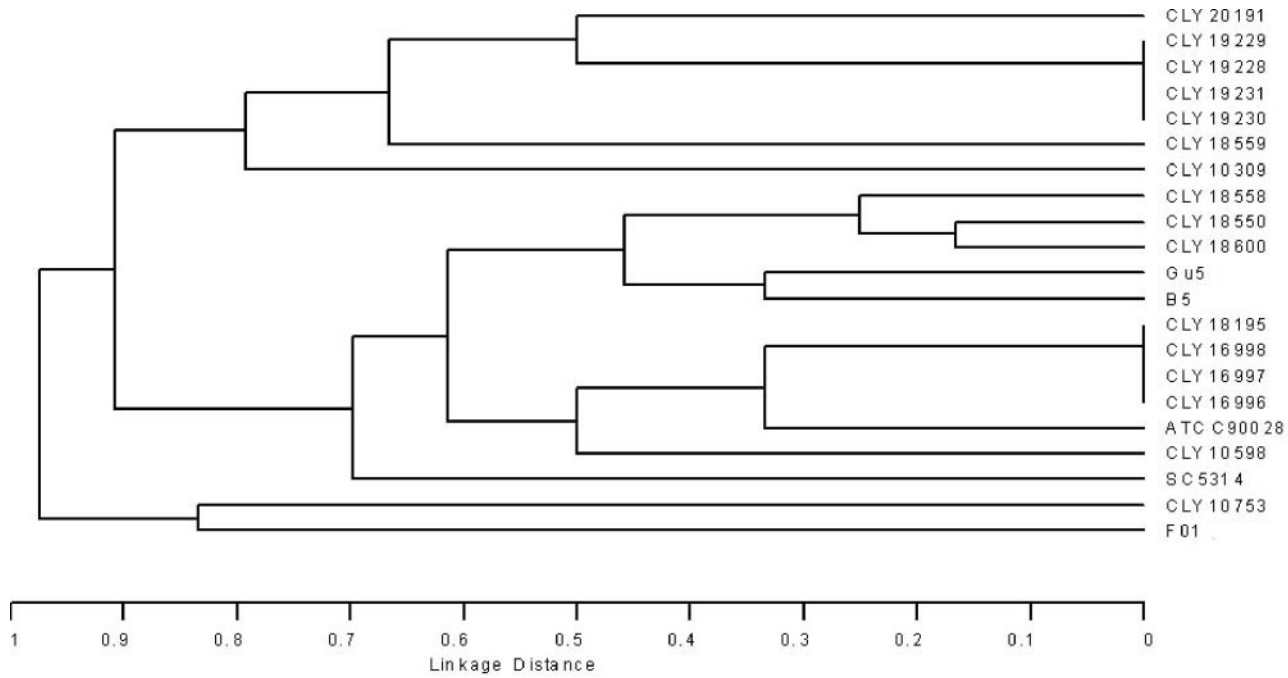

FIG. 5. Dendrogram and splits tree analysis of genetically related isolates. Dendrogram showing the genetic relatedness among 21 *C. albicans* isolates, per Robles et al. (36). The splits tree demonstrates the genetic relatedness among isolates. Sixteen strains with a CLY number from protocol 026 (including those described in Table 5) and five laboratory reference strains were evaluated.

in Ca*FKS1*. These strains were isolated from the liver and mouth, respectively (Table 5). MLST analysis based on six "housekeeping" genes was used to evaluate these four strains, along with other *C. albicans* clinical isolates. As shown in a dendrogram, the MLST signatures of all four strains from patient A are indistinguishable (Fig. 5). Analysis of four isolates from another patient (patient B; Table 5) provided similar results; the MLST patterns of strains CLY19230 and CLY19231, each with reduced susceptibility to caspofungin and a S645F Cafks1p substitution, and Ca*FKS1* wild-type strains CLY19228 and CLY19229 (caspofungin susceptible) were identical (Fig. 5).

In vitro frequency of spontaneous mutations conferring decreased caspofungin susceptibility. Clinical isolate CLY18195 represents a potential baseline isolate for the mutants that were recovered from patient A. It is possible that an inherently high incidence of mutations in strain CLY18195 accounts for the recovery of mutants in this patient. To address this, we measured the rate at which decreased susceptibility to caspofungin spontaneously developed in this strain by use of an in vitro fluctuation test. Among 20 replicate cultures, no spontaneous mutants grew on the selection plates. As a control, colonies were detected when cells of a *C. albicans* Ca*fks1* mutant strain (either CAI4-R1 or NR3) were added to individual cultures before they were spread on caspofungin-containing plates. We calculated a rate of $<2.56 \times 10^{-9}$ mutations per cell per generation, which was indistinguishable from the rate measured for another caspofungin-susceptible isolate ($<2.55 \times 10^{-9}$ mutations per cell per generation for *C. albicans* MY1055) (1). A rate of 2×10^{-8} mutations per cell division was reported in a previous study using a similar method with the echinocandin L-733560 and the laboratory isolate *C. albicans* CAI4 (22).

DISCUSSION

Echinocandins inhibit enzymatic synthesis of the critical cell wall polysaccharide 1,3-β-D glucan. The catalytic subunit of GS has not been definitively identified, and the mechanism of drug inhibition remains unclear. Mutations that confer reduced echinocandin susceptibility in *S. cerevisiae* and *C. albicans* map to *FKS1*, a gene encoding a large integral membrane protein which is a subunit of the GS complex (10–12, 22). Fks1p is the presumed target for echinocandins, and the *fks1* mutations are thought to alter drug binding and/or another event leading to inhibition. However, other potential interacting proteins such as Pilp and Lsp1 may play a role in the complex (13). The mechanistic nature of the interaction between echinocandins and GS remains to be elucidated.

This paper describes comprehensive genetic and biochemical studies of mutations in *FKS* genes that confer reduced echinocandin susceptibility to whole cells. Mutants with reduced drug susceptibility were identified from lab isolates of *S. cerevisiae* and *C. albicans* as well as clinical *Candida* isolates. *C. albicans* strains with these Ca*fks1* mutations are stable and display significantly higher ED_{90} values in a murine model of disseminated candidiasis. One group of amino acid substitutions, in the Fks proteins of *S. cerevisiae* (F639I, V641K, D646Y) and *C. albicans* (S645F, S645P, S645Y), maps to a short conserved region of ScFks1p and CaFks1p (Fig. 2). Four of these mutations were recreated in Sc*FKS1* by site-directed mutagenesis and analyzed in a Sc*fks1*Δ strain, confirming that each is both necessary and sufficient to confer reduced echinocandin susceptibility. Recently, a *S. cerevisiae* mutant with reduced susceptibility to the cyclic lipopeptide arborcandin C, which has a larger cyclic peptide nucleus and two lipophilic side chains (38), was shown to have an L642S substitution (31). This residue falls within the region of Fks1p identified in our work. Interestingly, the L642S mutation had little effect on the echinocandin susceptibility of either whole cells or GS derived from them (31).

We also showed that Ca*fks1h-1* (S645P) of CAI4-R1 reduces echinocandin susceptibility in *C. albicans* by demonstrating a significant reduction in sensitivity to L-733560 when Ca*fks1h-1* was integrated at either the *FKS1 or fks1* locus of a strain heterozygous for a disruption of Ca*FKS1*. These strains are similar to strain CAI4-RI, in that they contain one functional Ca*FKS1* gene and one functional Ca*fks1h-1* gene (Fig. 1) and show reduced susceptibility to L-733560. Integration of Ca*fks1h* at the Ca*FKS1* locus is necessary to construct a functional allele, since a nonhomologous integration would probably result in a noncontiguous gene. Indeed, the transformants in which Ca*fks1h-1* was not integrated at the Ca*FKS1* locus were as susceptible as the parent strain T1FOA (pJAM11).

The region between Phe 641 and Asp 648 of CaFks1p contains most of the mutations we found, including Ser645, which is the amino acid most often altered in our collection of laboratory and clinical *Candida* isolates with reduced echinocandin susceptibility. Clinical *C. albicans* isolates only showed changes at Ser645, although this observation likely reflects the small number of strains analyzed. This 8-amino-acid portion of Fks1p is part of a predicted 89-amino-acid-domain of Fks1p, which maps in some topology models to the cytoplasmic face of the plasma membrane (11). This domain has been proposed as the echinocandin binding site of 1,3-β-glucan synthase (25), although no direct evidence of binding to this or any other region of Fks1p has been reported. We also identified Arg 1361 of *C. krusei* Fks1p (and Arg 1357 of ScFks1p) as a site for substitutions in mutants with reduced echinocandin susceptibility. Arg 1361 of CaFks1p is predicted to be on the extracytoplasmic face of the protein, in close proximity to a transmembrane helix (11). It is not clear whether this residue directly affects drug susceptibility or whether it interacts in some manner with the other domain we have identified.

The development of a purification procedure for GS from *C. albicans* and *C. krusei* was critical for revealing the relationship between reduced whole-cell susceptibility and target-site inhibition. Caspofungin dose-response inhibition curves with purified GS reveal the presence or absence of wild-type and mutant Ca*FKS1* alleles (Fig. 4). In every case, significantly higher levels of caspofungin were necessary to inhibit GS from mutants with reduced echinocandin susceptibility. The kinetics for inhibition of wild-type GS and enzymes from laboratory strains containing only mutated Ca*fks1* allele(s) (e.g., S645Y/S645Y, S645P/null) reveals a classic single-site profile. In contrast, GS from heterozygous strains (e.g., S645P/WT) clearly displays a dual inflection point profile, reflecting both wild-type and mutant enzymes. It seems likely that wild-type and mutant enzymes behave independently and do not form a hybrid dimeric or multimeric enzyme.

Irrespective of a heterozygous or homozygous genotype, the presence of a mutant Ca*fks1* allele led to similarly high caspofungin MICs in liquid broth microdilution assays. However, these Ca*fks1* laboratory mutants could be distinguished by their response to caspofungin in an animal model of disseminated candidiasis. Among the laboratory isolates, the ED_{90} values for heterozygotes were at least 20-fold lower than those for homozygous (e.g., S645Y/S645Y) or pseudo-haploid (S645P/null) mutants. For clinical *Candida* isolates, the caspofungin GS inhibition curves and DNA sequence data are consistent with only homozygous mutations at the *fks1* locus. The caspofungin ED_{90} value for *C. albicans* strain CLY16997 (S645P/S645P), which was at least 70-fold higher than that of heterozygous laboratory mutants (strains CAI4-R1 and NR4; S645P/WT) with the same amino acid substitution, provides further evidence of homozygosity.

In a clinical study (41), multiple *C. albicans* strains were collected from different body sites of two patients and characterized. Two isolates from one patient (patient A) have reduced caspofungin susceptibility and distinct Ca*fks1* mutations (S645F or S645P), and two isolates have wild-type Ca*FKS1* genes and caspofungin susceptibility. The second patient (patient B) had two strains with identical Ca*fks1* mutations (S645F) and reduced susceptibility to caspofungin and two wild-type strains. MLST analysis demonstrated that each set of four isolates from a single patient was genetically indistinguishable from the others. Given the general diversity of clinical *C. albicans* isolates from different patients (Fig. 5), it is likely that the mutant strains recovered from these patients arose from a wild-type progenitor strain. We believe that these collections of strains reflect the emergence of isolates with a reduced caspofungin susceptibility phenotype under selective pressure. Furthermore, these results establish that strains with different *fks1* mutant alleles can be recovered from different sites within the same patient.

The Fks1p residues identified in this collection of mutants are conserved across diverse fungal genera (9), which suggests that these amino acids may play an important role in caspofungin susceptibility in many fungi. For example, the *C. krusei* clinical isolate CLY16038 contains an R1361G substitution in Fks1p; not only is Arg conserved at this position in most fungal pathogens, but the spontaneous *S. cerevisiae* MS14 mutant has a substitution at an equivalent Arg residue in ScFks1p. It is possible that fungi with weak intrinsic in vitro susceptibility to echinocandins (34) could have substitutions in their *FKS* genes which confer reduced GS susceptibility and account for the whole-cell phenotype. However, the CnFks1 protein from *Cryptococcus neoformans*, an organism that responds poorly to echinocandins in vitro and in vivo (1, 4), does not have any substitutions at the conserved Fksp residues identified in this report.

Our results indicate that target site amino acid substitutions in the GS Fks1p subunit are sufficient to confer reduced echinocandin susceptibility. The frequency at which spontaneous mutants arise in the laboratory is less than 10^{-9} mutations per cell per generation. DNA sequence analysis of six independent genes during MLST analysis did not show a statistical increase relative to Ca*FKS1* in spontaneous mutation rates in clinical strains with reduced susceptibility (data not shown). Similarly, the frequency of mutants in clinical experience has also been rare. To date, assessment of the caspofungin clinical trials database has identified only five isolates collected from three patients (all from protocol 026) with demonstrated evidence of reduced susceptibility to caspofungin. Despite a report suggesting that low-level shifts in susceptibility could be mediated by overexpression of the drug efflux transporter Cdr2p in *C. albicans* (37), we found no spontaneous mutants from either the clinical trial or our laboratory isolates whose phenotype was not explained by a target site mutation. Moreover, caspofungin is a poor substrate for multidrug efflux transporters in fluconazole-resistant strains of *C. albicans* expressing high levels of *CDR1*, *CDR2*, and/or *MDR1* (3, 39), and a recent survey of 351 fluconazole-resistant *Candida* isolates revealed that the organisms are inhibited by caspofungin at standard MIC_{90} doses (35). The *fks* mutants we characterized in this study maintained wild-type susceptibility to amphotericin B and fluconazole (references 12, 15, 22 and data not shown), which underscores the specificity of changes in GS for the echinocandin class of inhibitors. Molecular tools that could be used to rapidly identify high-probability changes in the Ca*FKS1* sequence may be very valuable for discriminating between different potential mechanisms of reduced drug susceptibility in *C. albicans* clinical isolates. We also envision that the *S. cerevisiae*, *C. albicans*, and *C. krusei fks1* mutants described in this work will be useful for characterizing interactions between 1,3-β-D glucan synthase and the echinocandins.

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