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Effect of *Candida glabrata FKS1* and *FKS2* Mutations on Echinocandin Sensitivity and Kinetics of 1,3-β-D-Glucan Synthase: Implication for the Existing Susceptibility Breakpoint▽

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Thirteen *Candida glabrata* strains harboring a range of mutations in hot spot regions of *FKS1* and *FKS2* were studied. The mutations were linked to an echinocandin reduced susceptibility phenotype. Sequence alignments showed that 11 out of the 13 mutants harbored a mutation in *FKS1* or *FKS2* not previously implicated in echinocandin reduced susceptibility in *C. glabrata*. A detailed kinetic characterization demonstrated that amino acid substitutions in Fks1p and Fks2p reduced drug sensitivity in mutant 1,3-β-D-glucan synthase by 2 to 3 log orders relative to that in wild-type enzyme. These mutations were also found to reduce the catalytic efficiency of the enzyme (V_{max}) and to influence the relative expression of *FKS* genes. In view of the association of *FKS* mutations and reduced susceptibility of 1,3-β-D-glucan synthase, an evaluation of the new CLSI echinocandin susceptibility breakpoint was conducted. Only 3 of 13 resistant *fks* mutants (23%) were considered anidulafungin or micafungin nonsusceptible (MIC > 2 μg/ml) by this criterion. In contrast, most *fks* mutants (92%) exceeded a MIC of >2 μg/ml with caspofungin. However, when MIC determinations were performed in the presence of 50% serum, all *C. glabrata fks* mutants showed MICs of ≥2 μg/ml for the three echinocandin drugs. As has been observed with *Candida albicans*, the kinetic inhibition parameter 50% inhibitory concentration may be a better predictor of *FKS*-mediated resistance. Finally, the close association between *FKS1*/*FKS2* hot spot mutations provides a basis for understanding echinocandin resistance in *C. glabrata*.

Candida glabrata is the second most-common fungal species isolated from blood in the United States and one of the most common fungal pathogens worldwide. *C. glabrata* infections are a concern due to their high mortality rates (38) and the tendency of this microorganism to rapidly develop resistance to azole antifungal agents (6, 11, 15, 25, 31, 34). The introduction of the antifungal drug caspofungin (CSF) in 2001 was a significant advance in the treatment of these infections since these lipopeptides' mode of action is independent of existing antifungal drugs (7, 16, 36). All three echinocandin drugs, CSF, anidulafungin (ANF), and micafungin (MCF), inhibit 1,3-β-D-glucan synthase, which disrupts the structure of the growing cell wall, causing osmotic instability and death of susceptible yeast cells (8, 22). 1,3-β-D-Glucan synthase is a protein complex formed at least by catalytic subunits (Fksp) and a ubiquitous regulatory element (Rho1). Echinocandin drugs are presumed to bind to Fksp, which is encoded by three putative *FKS* genes in *Candida* spp. and *Saccharomyces* spp. (*FKS1*, *FKS2*, and *FKS3*) (8, 9, 12, 17, 21). Echinocandin reduced susceptibility in *Candida* spp. has been linked to mutations in two highly conserved hot spot regions of *FKS* genes (22). Recently, three reports showed that amino acid substitutions in Fks1p (D632E) and Fks2p (F659V) are responsible for clinical echinocandin resistance in *C. glabrata* (3, 17, 33). The aim of this study was to investigate the linkage between echinocandin resistance and *FKS* mutations in a collection of clinical *C. glabrata* strains exhibiting reduced susceptibility to echinocandin drugs. Moreover, a detailed kinetic analysis was performed to assess the effect of the different amino acid substitutions on 1,3-β-D-glucan synthase complex kinetic parameters and inhibition by different echinocandin drugs. Finally, the new CLSI echinocandin susceptibility breakpoint was evaluated to establish its value in identifying echinocandin-resistant *C. glabrata* strains with *FKS* mutations.

MATERIALS AND METHODS

Strains. Fifteen clinical *C. glabrata* strains were used throughout this work. Two strains were susceptible to echinocandin drugs (strains 218 and 3168). The others showed CSF MICs of ≥2 μg/ml. Twelve of the 13 resistant strains were isolated from patients on CSF therapy or prophylaxis, while strain 234 was isolated after ANF therapy. Strain ATCC 90030 was used as wild-type control isolate. Strains 3168 (echinocandin susceptible) and 3169 (echinocandin resistant) are isogenic (3). The isolates were identified as *C. glabrata* by conventional microbiologic methods. The initial identification was confirmed by sequencing of the 5.8S RNA gene and adjacent internal transcribed spacer regions 1 and 2 (35). Molecular identification was performed in order to avoid misidentification with the *C. glabrata* novel anamorphic related species *Candida bracarensis* (5) and *Candida nivariensis* (1).

Antifungal susceptibility testing and compounds. Echinocandin drug susceptibility testing was performed in triplicate in accordance with the CLSI document M27-A3 guidelines (4) in the presence or absence of 50% human serum (Sigma-Aldrich) (20). *Candida parapsilosis* ATCC 22019 and *Candida krusei* ATCC 6258 were used as control strains for antifungal susceptibility testing. The drugs used were CSF (Merck & Co. Inc., Rahway, NJ), ANF (Pfizer, New York, NY), and MCF (Astellas Pharma USA, Inc., Deerfield, IL). The drugs were obtained as standard powders from their manufacturers. CSF and MCF were dissolved in sterile distilled water, and ANF was dissolved in 100% dimethyl sulfoxide (Sigma-Aldrich). Stock solutions of each drug were kept at −86°C.

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TABLE 1. In vitro whole-cell susceptibility (MIC) to and 1,3-β-D-glucan synthase inhibition profiles (IC_{50}) of echinocandin drugs for the strains included in the study

Strain	Genotype		Phenotype		MIC[c]						IC_{50}[d]		
					ANF		CSF		MCF				
	FKS1	*FKS2*	Fks1p[a]	Fks2p[b]	No serum	50% Serum	No serum	50% Serum	No serum	50% Serum	ANF	CSF	MCF
90030	WT	WT	WT	WT	0.06	0.50	0.06	0.12	0.03	0.25	1.91	1.92	0.71
218	WT	WT	WT	WT	0.06	0.50	0.12	0.25	0.12	1.00	1.25	3.11	0.56
3168	WT	WT	WT	WT	0.03	0.50	0.06	0.12	0.06	0.50	1.58	1.79	1.29
42997	T1874C	WT	F625S	WT	2.00	8.00	8.00	8.00	0.50	4.00	110.6	697.8	126.3
5847	T1885C	WT	S629P	WT	4.00	8.00	8.00	8.00	2.00	8.00	3855.4	4987.5	854.5
21900	A1895G	WT	D632G	WT	1.00	8.00	4.00	16.00	0.50	8.00	101.8	756.3	343.4
3169	T1896G	WT	D632E	WT	2.00	8.00	2.52	8.00	2.52	8.00	151.5	844.5	444.7
42971	G1894T	A4129T	D632Y	R1377STOP	4.00	8.00	8.00	8.00	1.00	8.00	2543.5	5248.5	413.4
31498	WT	Del1974–1976	WT	F659del	2.00	8.00	2.00	8.00	4.00	8.00	160	117.2	123.5
234	WT	T1975G	WT	F659V	1.00	8.00	4.00	8.00	1.00	8.00	86.6	115.3	113.5
41026	WT	T1976C	WT	F659S	4.00	16.00	16.00	16.00	4.00	16.00	858.1	1064.0	1067.0
3830	WT	T1987C	WT	S663P	1.00	8.00	16.00	16.00	1.00	8.00	4003.5	5115.5	2569
41400	WT	A1997G	WT	D666G	2.00	8.00	4.00	8.00	0.12	4.00	175.8	506.6	100.6
42031	WT	C1998A	WT	D666E	1.00	4.00	4.00	8.00	0.25	4.00	127.4	257.9	88.7
51916	WT	C1999A	WT	P667T	1.59	8.00	2.00	8.00	0.40	4.00	99.5	166.2	110.9
5416	WT	G4125A	WT	W1375L	0.25	4.00	4.00	8.00	0.25	2.00	85.2	608.4	50.7

[a] WT, wild type at hot spots. Fks1p hot spot number one includes amino acids between 625 and 633 (625-FLILSLRDP-633).
[b] WT, wild type at hot spots. Fks2p hot spot number one includes amino acids between 659 and 667 (659-FLILSLRDP-667). Fks2p hot spot number two comprises amino acids between 1374 and 1381 (WT, 1374- DWVRRYTL-1381).
[c] Geometric mean values in μg ml^{-1} (three repetitions on three separate days).
[d] Arithmetic mean (three repetitions on three separate days) IC_{50}s obtained using trapped 1,3-β-D-glucan synthase enzyme expressed in ng ml^{-1}.

***FKS* gene sequence analysis.** *C. glabrata* genomic DNA was extracted from yeast cells grown overnight in YPD (2% yeast extract, 4% Bacto peptone, 4% dextrose) broth medium with a Q-Biogene (Irvine, CA) FastDNA kit. PCR and sequencing primers were designed based on the *C. glabrata FKS1*, *FKS2*, and *FKS3* gene sequences (GenBank accession no. XM_446406, XM_448401, and XM_449945, respectively). DNA sequencing was performed with a CEQ dye terminator cycle sequencing quick start kit (Beckman Coulter, Fullerton, CA) according to the manufacturer's recommendations. Sequence analysis was performed with CEQ 8000 genetic analysis system software (Beckman Coulter, Fullerton, CA) and BioEdit sequence alignment editor (Ibis Therapeutics, Carlsbad, CA).

1,3-β-D-Glucan synthase isolation and assay. All isolates used in this work were grown with vigorous shaking at 35°C to early stationary phase in YPD 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 described previously (21). Sensitivity to echinocandin drugs was measured in a polymerization assay, using a 96-well multiscreen high-throughput screen filtration system (Millipore corporation, Bedford, MA) with a final volume of 100 μl (12, 21). Serial dilutions of echinocandin drugs (0.01 to 10,000 ng/ml) were used to determine 50% inhibitory concentration (IC_{50}) values. Control reactions were performed in the presence of 1% dimethyl sulfoxide when ANF was used. The reactions were initiated by addition of the purified 1,3-β-D-glucan synthase enzyme. Inhibition profiles and IC_{50} values were determined using a sigmoidal response (variable-slope) curve fitting algorithm with GraphPad Prism software (version 4.0; Prism Software, Irvine, CA).

Characterization of 1,3-β-D-glucan product. The product of the reaction mixtures was verified as 1,3-β-D-glucan by using a Glucatell kit (Associates of Cape Cod, Inc., Falmouth, MA), following the procedure previously described (12).

Kinetic analyses. All reactions were run in a 96-well multiscreen high-throughput screen filtration system (Millipore) with a final volume of 100 μl as previously described (12, 14). [^{3}H]uridine diphosphoglucose (UDPG) was used as the substrate in concentrations ranging from 0.015 to 2 mM to determine the different kinetic parameters, which were analyzed by linear regressions to obtain slopes in dpm/min. This value was then converted to nM of glucose incorporated per minute. The maximum velocity (V_{max}) and the Michaelis-Menten constant (K_m) were determined for trapped 1,3-β-D-glucan synthase complex by varying the amount of UDPG (between 0.015 and 2 mM) using Lineweaver-Burke plots.

RNA isolation and expression profiling. *C. glabrata* strains were grown in YPD at 37°C with shaking (150 rpm) for 16 h. Total RNA was extracted using an RNeasy mini kit (Qiagen), and gene expression profiles were performed using a one-step SYBR green quantitative reverse transcription-PCR kit (Stratagene, La Jolla, CA) with the Stratagene Mx3005P multiplex quantitative PCR system. Differential expression was analyzed for the three *C. glabrata FKS* genes. The relative expression levels were evaluated using the Pfaffl method (23). The *C. glabrata URA3* gene (GenBank accession no. AY771209) was used for normalization. The primers used for gene expression profiling were described previously (12).

Statistical analysis. The kinetic data are the result of experiments performed in triplicate. Arithmetic means and standard deviations were used to statistically analyze all the continuous variables (IC_{50}, K_m, and V_{max}). Geometric means were used to statistically compare MIC results. The significance levels of MIC differences and kinetic parameters were determined by Student's *t* test (unpaired, unequal variance); a *P* value of 0.05 was considered significant. In order to approximate a normal distribution, the MICs were transformed to $\log_2$ values to establish susceptibility differences between strains. Both on-scale and off-scale results were included in the analysis. The off-scale MICs were converted to the next concentration up or down. MICs were compared with IC_{50}s by using plots of the $\log_2$ MIC versus $\log_{10}$ IC_{50}. The MIC-IC_{50} breakpoint was defined as the MIC for which ≥90% of the strains had IC_{50}s for 1,3-β-D-glucan synthase at least 50-fold higher than that for the wild-type enzyme. Statistical analyses were performed with the Statistical Package for the Social Sciences software (version 13.0; SPSS, Inc., Chicago, IL).

RESULTS

Genetic analysis of *FKS* genes from echinocandin-resistant *C. glabrata* isolates. All *C. glabrata* strains exhibiting high echinocandin MICs showed mutations in either *FKS1* ($n = 4$), *FKS2* ($n = 8$), or both *FKS* genes ($n = 1$) (Table 1). Conversely, *FKS3* genes harbored only silent mutations compared with wild-type strain *FKS3* gene (data not shown). All of the mutations producing amino acid substitutions were found in one of the *FKS* mutational hot spots previously described for *Candida* spp. (3, 12, 14, 17, 21, 22, 33). Sequence alignments showed that 11 out of the 13 mutants in this collection harbor a mutation not previously implicated in echinocandin resistance in *C. glabrata*. The majority of the *FKS1* mutants showed an amino acid substitution at Asp632 (D632G, D632E, and D632Y), while the *FKS2* mutants harbor two dif-

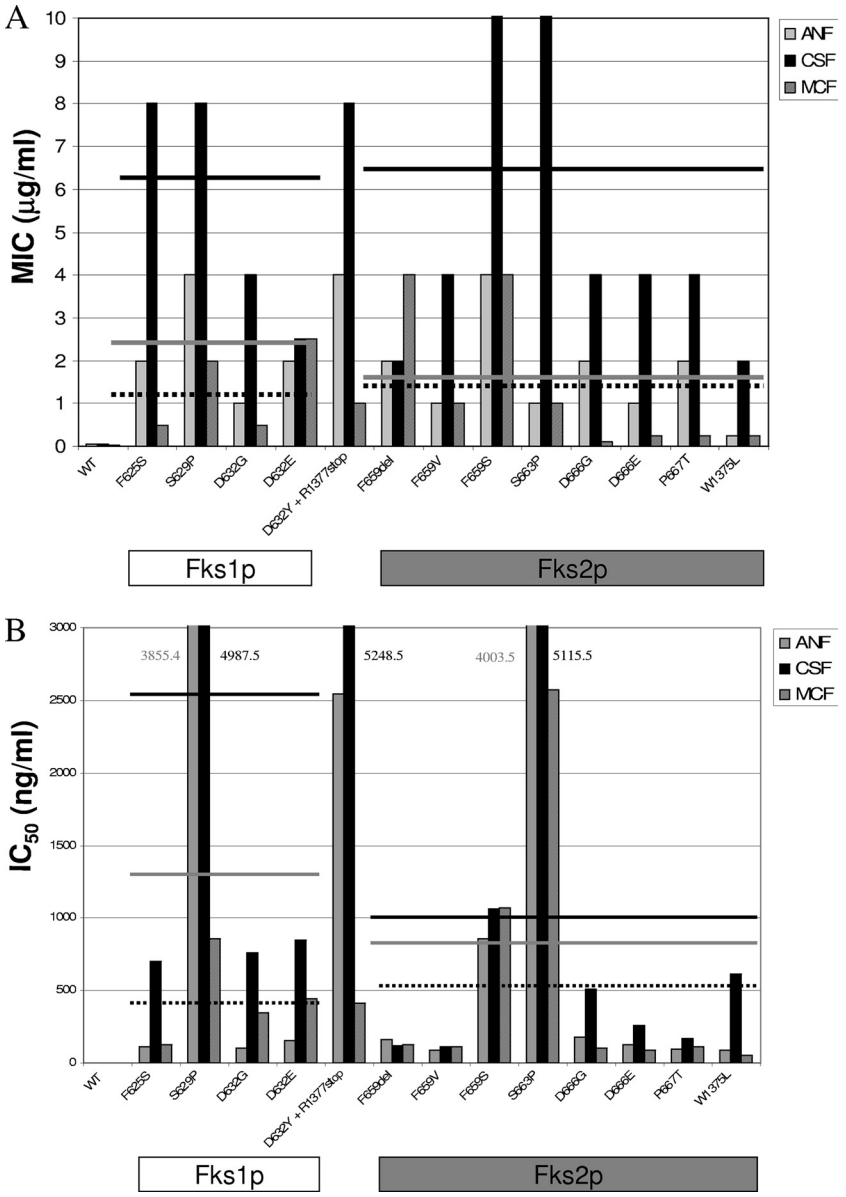


FIG. 1. Summary of echinocandin MIC (A) and IC_{50} (B) values for the *C. glabrata* strains harboring various Fks1p and Fks2p amino acid substitutions. Lines represent average MICs and IC_{50}s for ANF, CSF, and MCF for the group of mutants harboring amino acid substitutions in each Fks protein. MICs were obtained using the CLSI document M27-A3 guidelines, and IC_{50}s were obtained using product entrapped partially purified 1,3-β-D-glucan synthase enzyme complexes. WT, wild type.

ferent amino acid substitutions (F659V and F659S) and an amino acid deletion at Phe659 (F659del). The Phe659 deletion at Fks2p is due to a 3-nucleotide deletion (1974-CTT-1976), which maintains the same open reading frame but with a single frameshift. Strain 42971 is a double mutant with a mutation in *FKS1* (G1894T) and a nonsense mutation in *FKS2* producing a premature translation termination at codon Arg1377. This isolate and strain 5416 were the only two strains in the collection with an amino acid substitution at hot spot 2 (Fks2p).

Fks1p and Fks2p amino acid substitutions confer reduced echinocandin susceptibility in *C. glabrata*. All the *C. glabrata* strains with Fksp amino acid substitutions in at least one of the hot spots showed statistically significant reduced in vitro echinocandin susceptibility compared with that of wild type strains (40-fold [$P = 0.002$], 79-fold [$P = 0.005$], and 19-fold [$P = 0.039$] for ANF, CSF, and MCF, respectively). These differences were lower when MICs were obtained in the presence of serum (16-, 60-, and 12-fold for ANF, CSF and MCF, respectively). However, when serum was used, all the mutant strains showed MICs that surpassed the MIC susceptibility breakpoint proposed by the CLSI (4). All mutant strains exhibited statistically significant higher MICs for CSF than for the other drugs in the absence of serum ($P = 0.004$ for CSF versus ANF, and $P = 0.001$ for CSF versus MCF). On the other hand, MCF was the most potent of the three echinocandin drugs against *C. glabrata fks* mutants. These differences between drug potencies

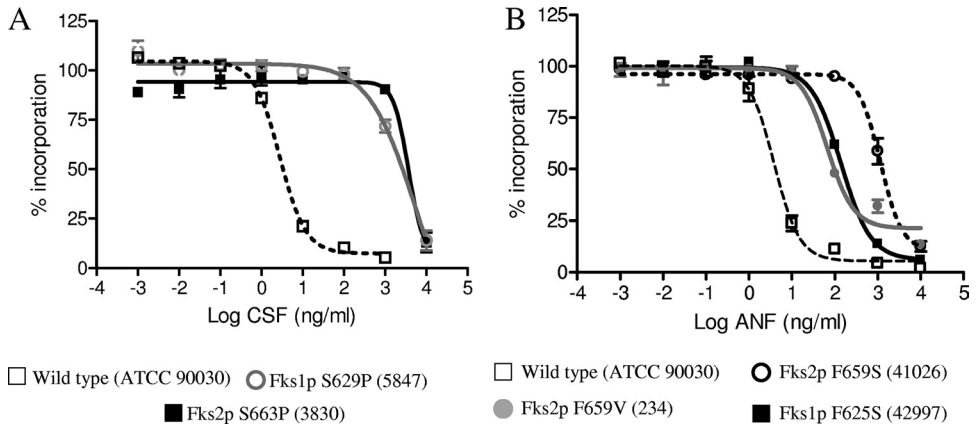


FIG. 2. Echinocandin inhibition profiles for product-entrapped 1,3-β-D-glucan synthase enzyme complexes assessed by the incorporation of [^{3}H]glucose into radiolabeled product. (A) CSF titration curves for enzymes isolated from strains ATCC 90030 (wild type), 5847 (Fks1p-S629P), and 3830 (Fks2p-S663P). (B) ANF inhibition curves for 1,3-β-D-glucan synthase enzyme complexes extracted from strains ATCC 90030 (wild type), 42997 (Fks1p-S625S), 234 (Fks2p-S659V), and 41026 (Fks2p-F659S).

disappear when the MICs were obtained in the presence of serum, as previously reported (19, 20, 37) ($P = 0.153$ and $P = 0.066$ when ANF and MCF, respectively, were compared with CSF) (Table 1). Echinocandin MICs varied with the specific mutations, but there was little difference in the average MICs between *FKS1* and *FKS2* mutants (Fig. 1). The highest MIC increases were seen with *C. glabrata* isolates harboring mutations F625S and S629P in Fks1p and the equivalent mutations in Fks2p, F659S and S663P (Table 1).

Effect of Fksp substitutions on 1,3-β-D-glucan synthase inhibition. The kinetic inhibition parameter IC_{50} was determined for 1,3-β-D-glucan synthase complexes isolated from *fks* mutant *C. glabrata* strains. Overall, the *fks* mutant enzymes showed significantly higher IC_{50}s than did corresponding enzymes isolated from wild-type strains (54-, 267-, and 60-fold on average for ANF, CSF, and MCF, respectively [$P < 0.001$ for all the drugs studied]). The CSF IC_{50}s were the highest of the three drugs (693-, 366-, and 577-fold increases for CSF, ANF and MCF, respectively) (Table 1). However, the pattern of 1,3-β-D-glucan synthase echinocandin susceptibility depends on the amino acid substitution and its localization (Table 1). Among the mutant 1,3-β-D-glucan synthase enzyme complexes studied, mutations at Ser629 (Fks1p) and Ser663 (Fks2p) showed the highest IC_{50}s for all echinocandin drugs ($P = 0.011$), followed by the F659S (Fks2p) (Table 1 and Fig. 2). Special consideration was required for the enzyme complex isolated from the strain 42971 (D632Y substitution in Fks1p coupled with an *FKS2* onsense mutation at nucleotide A4129T). This complex showed higher IC_{50}s for ANF and CSF than those obtained for the Fks1p D632G and D632E mutants (20- and sevenfold, respectively). However, there were no differences in MCF IC_{50}s between these three mutants suggesting that the Fks2p lack of function does not affect the echinocandin drug interaction with the complex. The Phe659 mutants (Fks2p) showed different IC_{50}s depending on which amino acid was replaced. The 1,3-β-D-glucan synthase complex harboring the F659S substitution in Fks2p showed echino-

TABLE 2. Summary of kinetic properties of the *C. glabrata* 1,3-β-D-glucan synthase complexes included in the study

Strain	Genotype		Phenotype		V_{max} (nmol min^{-1})[c]	K_m (mM)[c]
	FKS1	*FKS2*	Fks1p[a]	Fks2p[b]		
90030	WT	WT	WT	WT	7.04 ± 0.81	0.13 ± 0.02
218	WT	WT	WT	WT	6.85 ± 0.52	0.12 ± 0.04
3168	WT	WT	WT	WT	6.67 ± 0.18	0.10 ± 0.07
42997	T1874C	WT	F625S	WT	1.83 ± 0.59	0.17 ± 0.11
5847	T1885C	WT	S629P	WT	1.44 ± 0.95	0.15 ± 0.05
21900	A1895G	WT	D632G	WT	1.55 ± 0.41	0.10 ± 0.05
3169	T1896G	WT	D632E	WT	1.57 ± 0.38	0.07 ± 0.05
42971	G1894T	A4129T	D632Y	R1377STOP	1.26 ± 0.24	0.06 ± 0.01
31498	WT	Del1975–1977	WT	F659del	2.75 ± 0.22	0.10 ± 0.04
234	WT	T1975G	WT	F659V	2.87 ± 0.85	0.17 ± 0.04
41026	WT	T1976C	WT	F659S	3.05 ± 0.51	0.08 ± 0.01
3830	WT	T1987C	WT	S663P	4.45 ± 0.73	0.16 ± 0.05
41400	WT	A1997G	WT	D666G	5.05 ± 0.41	0.11 ± 0.03
42031	WT	C1998A	WT	D666E	1.85 ± 0.44	0.08 ± 0.01
51916	WT	C1999A	WT	P667T	0.33 ± 0.05	0.04 ± 0.02
5416	WT	G4125A	WT	W1375L	2.71 ± 0.09	0.04 ± 0.01

[a] WT, wild type at hot spots. Fks1p hot spot number one includes amino acids between 625 and 633 (625-FLILSLRDP-633).
[b] WT, wild type at hot spots. Fks2p hot spot number one includes amino acids between 659 and 667 (659-FLILSLRDP-667).
[c] Arithmetic mean ± standard deviation (three repetitions).

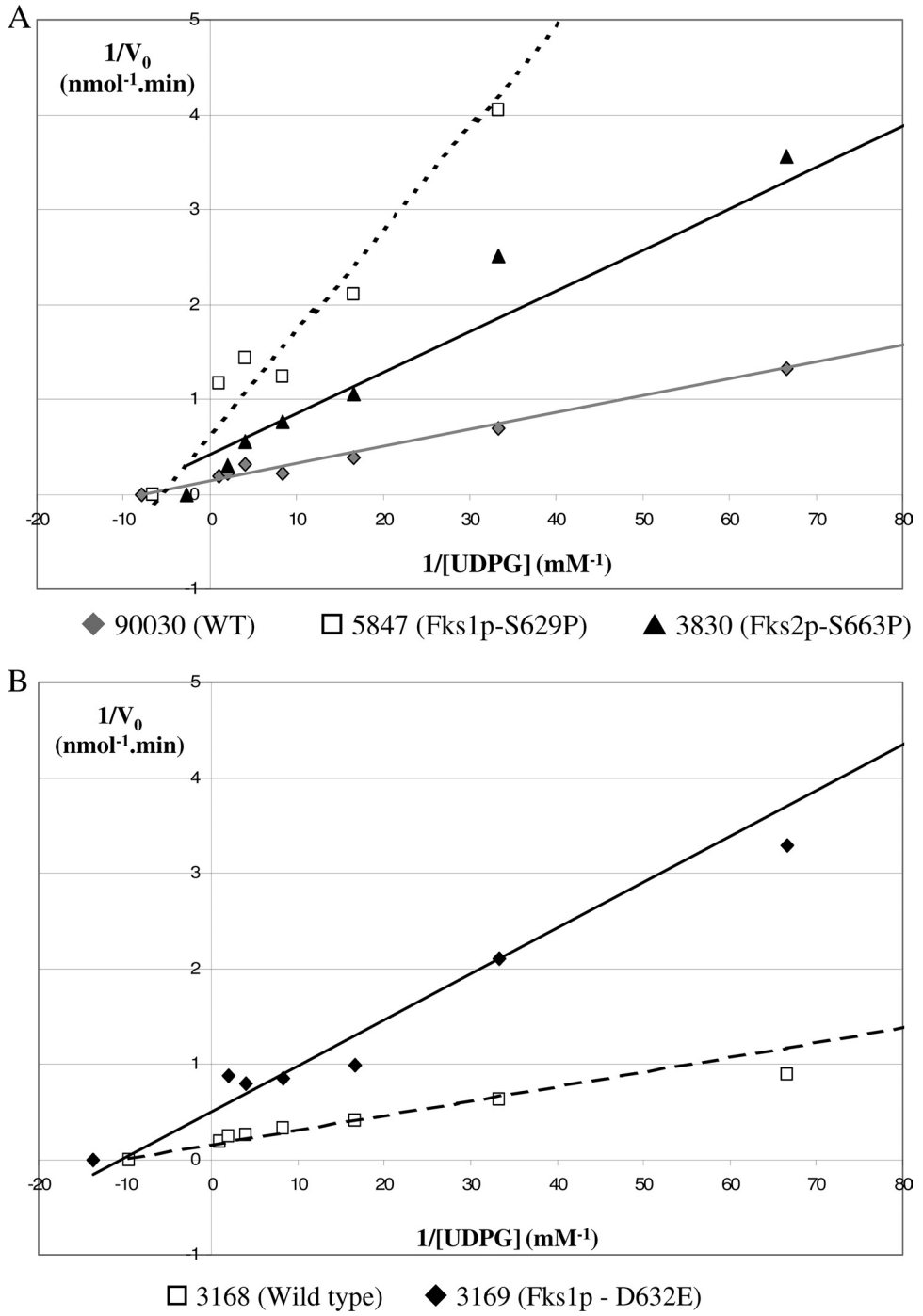


FIG. 3. Lineweaver-Burke double-reciprocal plots used to determine the maximum velocity (V_{max}) and the Michaelis-Menten constant (K_m) for product-entrapped 1,3-β-D-glucan synthase complexes by varying the amount of [^{3}H]UDPG. (A) Comparison of the kinetic properties of the 1,3-β-D-glucan synthase enzymes isolated from strains ATCC 90030 (wild type [WT]), 5847 (Fks1p-S629P), and 3830 (Fks2p-S663P). (B) Evaluation of the kinetic properties of the 1,3-β-D-glucan synthase enzyme obtained from the isogenic strains 3168 (WT) and 3169 (Fks1p-D632G).

candin IC_{50}s 10-fold higher than those of the F659V and the F659del mutant enzymes (Table 1 and Fig. 2). Finally, amino acid substitutions in the C termini of hot spot 1 (strain 51916) and hot spot 2 (strain 5416) showed the lowest IC_{50}s among the collection, indicating that these enzymes were the most sensitive to drug, which was also reflected in MICs.

***C. glabrata* 1,3-β-D-glucan synthase enzyme kinetics.** The kinetic parameters V_{max} and K_m for wild-type enzymes ($n = 3$) showed average values of 6.812 ± 0.246 nmol/min and 0.133 ± 0.015 mM, respectively (Table 2). Among the mutant enzyme complexes, amino acid substitutions in the hot spot regions generally decreased the catalytic capacity of the mutant en-

zyme V_{max} (on average, 2.380 ± 0.375 nmol/min; $P < 0.001$ relative to wild-type enzyme). On the other hand, K_m values for mutant enzymes were not statistically different than wild-type enzymes ($P = 0.660$). Amino acid substitutions that alter echinocandin sensitivity decrease enzyme velocity, but they do not change the binding affinity (K_m) of the enzyme for echinocandin drugs. These data are consistent with the noncompetitive kinetic behavior of echinocandin drugs on substrate, as described previously (14, 32). The extent of the V_{max} reduction depends on the amino acid substitution and its localization. The Fks1p mutant enzymes showed V_{max} values lower than those of Fks2p mutant enzymes (arithmetic means ± standard deviations were 1.530 ± 0.208 nmol/min for Fks1p mutants and 2.883 ± 1.455 nmol/min for Fks2p mutants; $P = 0.008$) (Table 2 and Fig. 3). Fks1p mutant enzymes showed a uniform V_{max} reduction with respect to wild-type strains (4.55-fold lower on average). On the other hand, Fks2p mutants showed a wider range of V_{max} reduction with the highest V_{max} values obtained from strains 3830 and 41400, Fks2p-S663P, and Fks2-D666G, respectively, and the lowest V_{max} from strain 51916, Fks2p-P667T. These data demonstrate that a single *FKS* mutation at one hot spot locus is sufficient to reduce the V_{max} of the *C. glabrata* 1,3-β-D-glucan synthase complex. This view was further supported when the 1,3-β-D-glucan synthase complexes from isogenic strains 3168 and 3169 were shown to have a V_{max} reduction of more than threefold (6.55 ± 0.38 nmol/min and 1.70 ± 0.32 nmol/min, respectively) (Table 2 and Fig. 3).

***C. glabrata FKS* gene expression varies with genotype.** SYBR green real-time PCR was used to evaluate the transcription levels of the three different *C. glabrata FKS* genes relative to *C. glabrata URA3* gene. The *FKS1*/*FKS2* ratios suggested that *FKS2* is expressed in a proportion ranging between 2:1 to 3:1 with respect to *FKS1* in the wild type ($n = 3$) and in the majority of the *FKS2* mutants ($n = 6$). *FKS3* was expressed at a very low level (750-fold lower expression on average) compared to both *FKS1* and *FKS2* in the complete collection (Table 3). All of the *FKS1* mutants ($n = 4$), one *FKS2* mutant (strain 51916; Fks2p P667T), and the double mutant (strain 42971; Fks1p D632Y and truncated Fks2p at the amino acid 1377) showed *FKS1*/*FKS2* ratios of >1. Despite the similar increases in expression ratios in these strains, there were differences found for *FKS1* and *FKS2* expression values relative to those of the wild type (strain 90030). The *FKS1* expression values were significantly higher in the *FKS1* mutants than in the rest of the collection ($P = 0.01$) (data not shown), demonstrating that the higher *FKS1*/*FKS2* ratios in *FKS1* mutants are due to changes in the expression of *FKS1*. On the other hand, strains 51916 and 42971 were the only isolates showing a significant *FKS2* reduction relative to *FKS2* expression of wild-type strains. Collectively, these data support the notion that the expression of the *FKS* genes is regulated by the relative functionality of each of the components of the 1,3-β-D-glucan synthase complex.

TABLE 3. Relative expression ratios between *FKS* genes

Strain	Genotype		Expression ratio[c]	
	FKS1[a]	*FKS2*[b]	*FKS1*/*FKS2*	*FKS3*/*FKS2*
90030	WT	WT	0.65	0.01
218	WT	WT	0.49	0.04
3168	WT	WT	0.67	0.06
42997	F625S	WT	1.79	0.07
5847	S629P	WT	1.95	0.01
21900	D632G	WT	1.30	0.13
3169	D632E	WT	1.20	0.07
42971	D632Y	R1377STOP	7.84	0.05
31498	WT	F659del	0.49	0.06
234	WT	F659V	0.95	0.07
41026	WT	F659S	0.29	0.01
3830	WT	S663P	0.58	0.05
41400	WT	D666G	0.85	0.06
42031	WT	D666E	0.42	0.05
51916	WT	P667T	1.23	0.01
5416	WT	W1375L	0.68	0.06

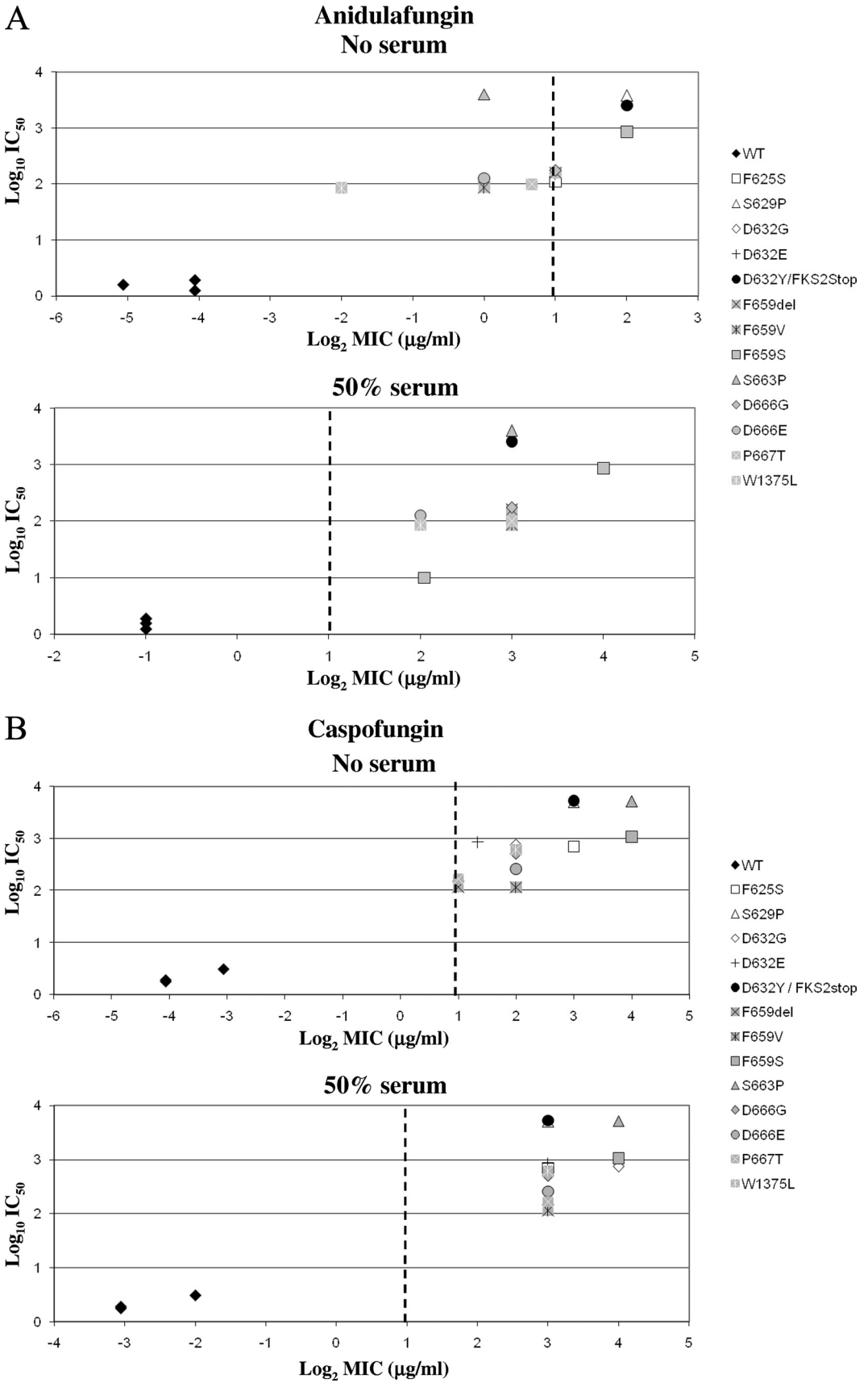
[a] WT, wild type at hot spots. Fks1p hot spot number one includes amino acids between 625 and 633 (625-FLILSLRDP-633).
[b] WT, wild type at hot spots. Fks2p hot spot number one includes amino acids between 659 and 667 (659-FLILSLRDP-667).
[c] Values are arithmetic means (three repetitions).

DISCUSSION

***Candida glabrata FKS1* and *FKS2* mutations are linked to echinocandin resistance.** Large-scale surveillance studies have documented the outstanding potency of echinocandin drugs against clinical isolates of *C. glabrata*, using the CLSI reference method (10, 24, 25, 27). Clinical failures are uncommon, although reports of echinocandin resistance with *C. glabrata* are emerging (3, 17, 33). In these studies, amino acid substitutions in both Fks proteins (Fks1p-D632E and Fks2p-F659V) have been linked with echinocandin reduced susceptibilities. The data presented here from a collection of *C. glabrata* strains with reduced echinocandin susceptibility, including some isolated after therapeutic failure, confirm that mutations in both *FKS* genes are associated with echinocandin resistance. Moreover, the treatment with ANF (strain 234) or CSF (all *FKS* mutants, excluding strain 234) were shown to have no influence on the MICs and IC_{50}s obtained (Table 1).

We also demonstrate that a range of mutations in *FKS1* and *FKS2* are responsible for echinocandin reduced susceptibility and clinical failures in *C. glabrata*, including 11 new *FKS* mutations. In contrast to *FKS1* and *FKS2*, *FKS3* appears to have no influence on the echinocandin susceptibility/resistance phenotype in *C. glabrata*. All *FKS* mutations described in this work were shown to be linked with increases in echinocandin MIC and IC_{50}, supporting the contention that changes in *FKS* is a universal echinocandin resistance mechanism among *Candida* spp. (12–14, 17, 22). It has to be noted that the *FKS* mutants studied in this work do not represent the frequency of the mutations in *C. glabrata FKS*. These mutants were selected to represent a variety of *FKS* mutations and were pulled out from a larger collection of isolates with high echinocandin MICs. The real frequency of each of the mutations should be obtained using a prospective population-based surveillance study.

It should be noted that 1,3-β-D-glucan synthase complexes isolated from *C. glabrata* have two Fksp isoenzymes. In the majority of the cases, only one of the Fksp proteins showed an amino acid substitution. However, the IC_{50} and kinetic profiles did not reveal the presence of mixed enzyme species (Fig. 2 and 3), suggesting that the Fksp proteins may form a multimeric catalytic functional subunit that behaves as a single kinetic entity. Thus, a mutation in one of the Fksp subunits alters


A
Anidulafungin
No serum
Log10 IC50
Log2 MIC (μg/ml)
50% serum
Log10 IC50
Log2 MIC (μg/ml)
WT
F625S
S629P
D632G
D632E
D632Y/FKS2Stop
F659del
F659V
F659S
S663P
D666G
D666E
P667T
W1375L
B
Caspofungin
No serum
Log10 IC50
Log2 MIC (μg/ml)
50% serum
Log10 IC50
Log2 MIC (μg/ml)
WT
F625S
S629P
D632G
D632E
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F659del
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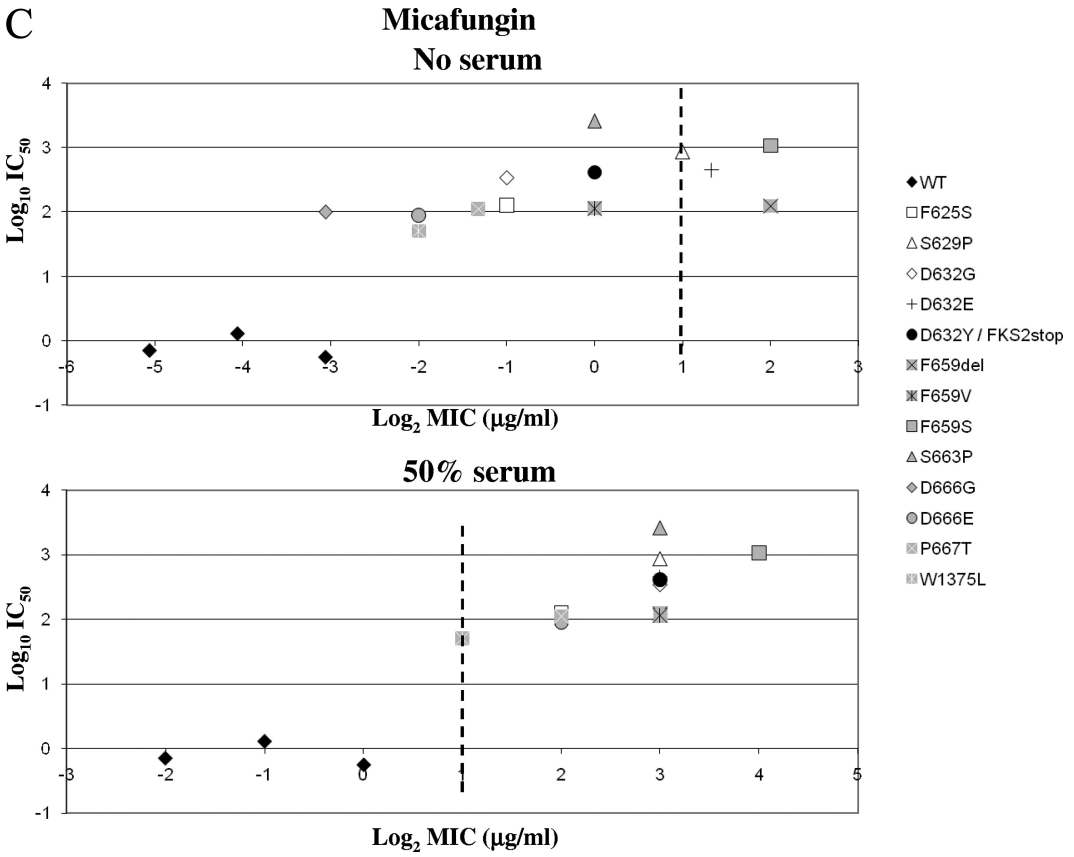

FIG. 4. MIC/IC_{50} correlation as a function of Fksp amino acid substitutions. The echinocandin drug ANF (A), CSF (B), or MCF (C) was used to obtain IC_{50} values and MICs in the presence of RPMI 1640 medium (CLSI document M27-A3) (4) or RPMI 1640 medium with 50% serum. Vertical dashed lines represent the CLSI susceptibility breakpoint (2 μg/ml). WT, wild type.

the kinetic profiles of the complete 1,3-β-D-glucan synthase complex.

CLSI echinocandin susceptibility breakpoints underrepresent echinocandin-resistant *C. glabrata* harboring *FKS* mutations. The CLSI has established an echinocandin MIC breakpoint for susceptibility of ≤2 μg/ml for *Candida* spp. to all three echinocandin drugs. This proposed breakpoint was based on microbiologic in vitro susceptibility data obtained from large-scale global surveillance, in vivo outcomes, and pharmacokinetic and pharmacodynamic studies (4, 26). In this study, all but one of the isolates were obtained as a result of CSF clinical failure; only 3 out of 13 *C. glabrata fks* clinical isolates (23%) could be considered ANF or MCF nonsusceptible using the CLSI breakpoint. In contrast, most of the *fks* mutants (92%) were CSF nonsusceptible. Yet, the biochemical sensitivity (IC_{50}) of all the mutant 1,3-β-D-glucan synthase enzymes (n = 13) was consistent with a less susceptible enzyme, which showed at least 50-fold higher IC_{50}s for all drugs than those with wild-type enzyme (Fig. 4). These IC_{50}s were comparable with previous data obtained for other echinocandin-resistant *Candida* species strains with *FKS* mutations (13, 14, 21, 22).

The MIC susceptibility breakpoint appears to overstate the susceptibility endpoint for *C. glabrata* strains with *FKS* mutations. This is largely because of the low number of *C. glabrata* clinical isolates with MICs of >2 μg/ml that were available at the time the breakpoint was established (only 1 out of 747 *C. glabrata* strains used in the study) (26). Moreover, the molecular mechanism of echinocandin resistance was not strictly considered. Given these limitations, it was appropriately reasoned that there was insufficient data to assign a resistance breakpoint (4, 26). While all three echinocandin drugs share the same target, in vivo potency, mechanism of resistance, and spectrum, they vary in antifungal properties in the CLSI testing medium. In the absence of serum, ANF and MCF appear to be more potent than CSF. However, these relative differences in potency are effectively minimized in the presence of 50% serum and confirmed in in vivo efficacy models (19, 20, 37). In the presence of serum, all of the *C. glabrata fks* mutant strains show significant shifts in MIC, which place them outside the susceptibility breakpoint, while all wild-type strains are maintained within the breakpoint MIC (Table 1 and Fig. 4).

There is an unambiguous linkage between increased echinocandin IC_{50}s, *FKS* mutations, and treatment failure. Yet, there is no clear linkage between ANF and MCF MICs and Fksp amino acid substitutions. The data presented here suggest several approaches to improve the relationship between MIC breakpoint, *FKS* mutations, and the potential for successful clinical outcome. One is to reduce the ANF and MCF breakpoints (>0.25 μg/ml) in order to distinguish between *C. glabrata FKS* mutants and wild-type strains. The second is the modification of the CLSI protocol to include 50% serum to the MIC plates. However, this latter idea has some disadvantages

regarding safety and standardization difficulties. A third is to use CSF as a surrogate marker to predict the *C. glabrata* susceptibility to the other echinocandin drugs, as previously suggested for fluconazole and azole susceptibility (28–30). Finally, the fourth is to use advances in molecular detection to identify specific *fks* mutations as resistance markers for *Candida* spp.

***FKS* mutations influence 1,3-β-D-glucan synthase kinetics and *FKS* gene expression.** Specific hot spot Fks amino acid substitutions were shown to decrease the V_{max} of the enzyme complex (Table 2). This lower catalytic capacity may have a potential fitness cost to the cell, as suggested by our group for other *Candida* spp. (12–14), which may contribute to the relatively low frequency of echinocandin resistance. In the case of *C. albicans* and *C. parapsilosis*, the reduction in V_{max} could be compensated for by an increase in *FKS2* expression. The data presented here confirm that modulation of the relative expression of *FKS* genes is a common compensatory response when the overall catalytic potential of the 1,3-β-D-glucan synthase complex is compromised. However, in *C. glabrata*, the expression of level of *FKS1* is mostly modulated in response to Fks1p amino acid substitutions. For most isolates, the level of *FKS2* transcript remained constant relative to that of *URA3*, irrespective of whether mutations occurred in *FKS1* or *FKS2*. The modulation of *FKS1* was most apparent in the behavior of isogenic wild-type and *fks1* mutant strains 3168 and 3169 in which a V_{max} decrease (Table 2) was manifested as a relative increase in *FKS1* expression (Table 3). The third gene, *FKS3*, is expressed at a trace amount and does not appear to play a role in 1,3-β-D-glucan synthase action; it is unaffected by mutations in *FKS1* and *FKS2*.

In our collection, there were two strains with mutations in *FKS2* showing *FKS1*/*FKS2* ratios of >1: strains 51916 (Fks2p-P667T) and 42971 (Fks1p-D632Y and Fks2p early termination). In both cases, a decrease in *FKS2* expression was observed. Strain 51916 showed one of the lowest IC_{50} and V_{max} values in this collection. These kinetic parameters are consistent with those obtained for *C. parapsilosis*, *Candida metapsilosis*, and *Candida orthopsilosis*, which harbor a naturally occurring amino acid substitution in the equivalent position of Fks1p (12). However, this strain showed an *FKS1*/*FKS2* ratio comparable to those obtained for *FKS1* mutants, suggesting that this particular strain has an unknown alteration in *FKS2* regulation. With respect to the strain with the Fks2p early termination codon (42971), the significant decrease in *FKS2* expression may be produced by a nonsense-mediated mRNA decay. This is a natural cell mechanism to ensure the elimination of incomplete processed mRNA and has been described for various eukaryotic cells, including *Saccharomyces cerevisiae* (2, 18, 39).

In summary, mutations in *FKS* genes of *C. glabrata* that yield 1,3-β-D-glucan synthase enzymes with highly reduced sensitivity to echinocandin drugs result in elevated MICs with a strong potential for clinical failure. The existing CLSI breakpoint does not adequately cover all three echinocandin drugs and may require modification for suitable capture. Finally, the cell senses changes in the catalytic behavior of 1,3-β-D-glucan synthase, which influences the relative expression of *FKS* genes.

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REFERENCES

1. **Alcoba-Florez, J., S. Mendez-Alvarez, J. Cano, J. Guarro, E. Perez-Roth, and A. M. del Pilar.** 2005. Phenotypic and molecular characterization of *Candida nivariensis* sp. nov., a possible new opportunistic fungus. J. Clin. Microbiol. **43:**4107–4111.
2. **Amrani, N., M. S. Sachs, and A. Jacobson.** 2006. Early nonsense: mRNA decay solves a translational problem. Nat. Rev. Mol. Cell Biol. **7:**415–425.
3. **Cleary, J. D., G. Garcia-Effron, S. W. Chapman, and D. S. Perlin.** 2008. Reduced *Candida glabrata* susceptibility secondary to an *FKS1* mutation developed during candidemia treatment. Antimicrob. Agents Chemother. **52:**2263–2265.
4. **Clinical Laboratory Standards Institute.** 2008. Reference method for broth dilution antifungal susceptibility testing of yeasts. Approved standard M27-A3, 3rd ed. Clinical Laboratory Standards Institute, Wayne, PA.
5. **Correia, A., P. Sampaio, S. James, and C. Pais.** 2006. *Candida bracarensis* sp. nov., a novel anamorphic yeast species phenotypically similar to *Candida glabrata.* Int. J. Syst. Evol. Microbiol. **56:**313–317.
6. **Cuenca-Estrella, M., A. Gomez-Lopez, E. Mellado, G. Garcia-Effron, and J. L. Rodriguez-Tudela.** 2004. In vitro activities of ravuconazole and four other antifungal agents against fluconazole-resistant or -susceptible clinical yeast isolates. Antimicrob. Agents Chemother. **48:**3107–3111.
7. **Denning, D. W.** 2003. Echinocandin antifungal drugs. Lancet **362:**1142–1151.
8. **Douglas, C. M.** 2001. Fungal β(1,3)-D-glucan synthesis. Med. Mycol. **39**(Suppl. 1)**:**55–66.
9. **Douglas, C. M., F. Foor, J. A. Marrinan, N. Morin, J. B. Nielsen, A. M. Dahl, P. Mazur, W. Baginsky, W. Li, and M. el Sherbeini.** 1994. The *Saccharomyces cerevisiae FKS1* (ETG1) gene encodes an integral membrane protein which is a subunit of 1,3-β-D-glucan synthase. Proc. Natl. Acad. Sci. USA **91:**12907–12911.
10. **Espinel-Ingroff, A.** 2003. In vitro antifungal activities of anidulafungin and micafungin, licensed agents and the investigational triazole posaconazole as determined by NCCLS methods for 12,052 fungal isolates: review of the literature. Rev. Iberoam. Micol. **20:**121–136.
11. **Fidel, P. L., Jr., J. A. Vazquez, and J. D. Sobel.** 1999. *Candida glabrata*: review of epidemiology, pathogenesis, and clinical disease with comparison to *C. albicans*. Clin. Microbiol. Rev. **12:**80–96.
12. **Garcia-Effron, G., S. K. Katiyar, S. Park, T. D. Edlind, and D. S. Perlin.** 2008. A naturally occurring proline-to-alanine amino acid change in Fks1p in *Candida parapsilosis*, *Candida orthopsilosis*, and *Candida metapsilosis* accounts for reduced echinocandin susceptibility. Antimicrob. Agents Chemother. **52:**2305–2312.
13. **Garcia-Effron, G., D. P. Kontoyiannis, R. E. Lewis, and D. S. Perlin.** 2008. Caspofungin-resistant *Candida tropicalis* strains causing breakthrough fungemia in patients at high risk for hematologic malignancies. Antimicrob. Agents Chemother. **52:**4181–4183.
14. **Garcia-Effron, G., S. Park, and D. S. Perlin.** 2009. Correlating echinocandin MIC and kinetic inhibition of *fks1* mutant glucan synthases for *C. albicans*: implications for interpretive breakpoints. Antimicrob. Agents Chemother. **53:**112–122.
15. **Hachem, R., H. Hanna, D. Kontoyiannis, Y. Jiang, and I. Raad.** 2008. The changing epidemiology of invasive candidiasis: *Candida glabrata* and *Candida krusei* as the leading causes of candidemia in hematologic malignancy. Cancer **112:**2493–2499.
16. **Kartsonis, N. A., J. Nielsen, and C. M. Douglas.** 2003. Caspofungin: the first in a new class of antifungal agents. Drug Resist. Updat. **6:**197–218.
17. **Katiyar, S., M. Pfaller, and T. Edlind.** 2006. *Candida albicans* and *Candida glabrata* clinical isolates exhibiting reduced echinocandin susceptibility. Antimicrob. Agents Chemother. **50:**2892–2894.
18. **Kerenyi, Z., Z. Merai, L. Hiripi, A. Benkovics, P. Gyula, C. Lacomme, E. Barta, F. Nagy, and D. Silhavy.** 2008. Inter-kingdom conservation of mechanism of nonsense-mediated mRNA decay. EMBO J. **27:**1585–1595.
19. **Odabasi, Z., V. Paetznick, J. H. Rex, and L. Ostrosky-Zeichner.** 2007. Effects of serum on in vitro susceptibility testing of echinocandins. Antimicrob. Agents Chemother. **51:**4214–4216.
20. **Paderu, P., G. Garcia-Effron, S. Balashov, G. Delmas, S. Park, and D. S. Perlin.** 2007. Serum differentially alters the antifungal properties of echinocandin drugs. Antimicrob. Agents Chemother. **51:**2253–2256.
21. **Park, S., R. Kelly, J. N. Kahn, J. Robles, M. J. Hsu, E. Register, W. Li, V. Vyas, H. Fan, G. Abruzzo, A. Flattery, C. Gill, G. Chrebet, S. A. Parent, M. Kurtz, H. Teppler, C. M. Douglas, and D. S. Perlin.** 2005. Specific substitutions in the echinocandin target Fks1p account for reduced susceptibility of rare laboratory and clinical *Candida* sp. isolates. Antimicrob. Agents Chemother. **49:**3264–3273.

22. **Perlin, D. S.** 2007. Resistance to echinocandin-class antifungal drugs. Drug Resist. Updat. **10:**121–130.
23. **Pfaffl, M. W.** 2001. A new mathematical model for relative quantification in real-time RT-PCR. Nucleic Acids Res. **29:**e45.
24. **Pfaller, M. A., L. Boyken, R. J. Hollis, S. A. Messer, S. Tendolkar, and D. J. Diekema.** 2006. Global surveillance of in vitro activity of micafungin against *Candida*: a comparison with caspofungin by CLSI-recommended methods. J. Clin. Microbiol. **44:**3533–3538.
25. **Pfaller, M. A., and D. J. Diekema.** 2002. Role of sentinel surveillance of candidemia: trends in species distribution and antifungal susceptibility. J. Clin. Microbiol. **40:**3551–3557.
26. **Pfaller, M. A., D. J. Diekema, L. Ostrosky-Zeichner, J. H. Rex, B. D. Alexander, D. Andes, S. D. Brown, V. Chaturvedi, M. A. Ghannoum, C. C. Knapp, D. J. Sheehan, and T. J. Walsh.** 2008. Correlation of MIC with outcome for *Candida* species tested against caspofungin, anidulafungin, and micafungin: analysis and proposal for interpretive MIC breakpoints. J. Clin. Microbiol. **46:**2620–2629.
27. **Pfaller, M. A., S. A. Messer, L. Boyken, C. Rice, S. Tendolkar, R. J. Hollis, and D. J. Diekema.** 2003. Caspofungin activity against clinical isolates of fluconazole-resistant *Candida*. J. Clin. Microbiol. **41:**5729–5731.
28. **Pfaller, M. A., S. A. Messer, L. Boyken, C. Rice, S. Tendolkar, R. J. Hollis, and D. J. Diekema.** 2004. Cross-resistance between fluconazole and ravuconazole and the use of fluconazole as a surrogate marker to predict susceptibility and resistance to ravuconazole among 12,796 clinical isolates of *Candida* spp. J. Clin. Microbiol. **42:**3137–3141.
29. **Pfaller, M. A., S. A. Messer, L. Boyken, C. Rice, S. Tendolkar, R. J. Hollis, and D. J. Diekema.** 2007. Use of fluconazole as a surrogate marker to predict susceptibility and resistance to voriconazole among 13,338 clinical isolates of *Candida* spp. tested by Clinical and Laboratory Standards Institute-recommended broth microdilution methods. J. Clin. Microbiol. **45:**70–75.
30. **Pfaller, M. A., S. A. Messer, L. Boyken, S. Tendolkar, R. J. Hollis, and D. J. Diekema.** 2008. Selection of a surrogate agent (fluconazole or voriconazole) for initial susceptibility testing of posaconazole against *Candida* spp.: results from a global antifungal surveillance program. J. Clin. Microbiol. **46:**551–559.
31. **Safdar, A., V. Chaturvedi, B. S. Koll, D. H. Larone, D. S. Perlin, and D. Armstrong.** 2002. Prospective, multicenter surveillance study of *Candida glabrata*: fluconazole and itraconazole susceptibility profiles in bloodstream, invasive, and colonizing strains and differences between isolates from three urban teaching hospitals in New York City (*Candida* Susceptibility Trends Study, 1998 to 1999). Antimicrob. Agents Chemother. **46:**3268–3272.
32. **Tang, J., and T. R. Parr, Jr.** 1991. W-1 solubilization and kinetics of inhibition by cilofungin of *Candida albicans* (1,3)-β-D-glucan synthase. Antimicrob. Agents Chemother. **35:**99–103.
33. **Thompson, G. R., III, N. P. Wiederhold, A. C. Vallor, N. C. Villareal, J. S. Lewis, and T. F. Patterson.** 2008. Development of caspofungin resistance following prolonged therapy for invasive candidiasis secondary to *Candida glabrata* infection. Antimicrob. Agents Chemother. **52:**3783–3785.
34. **Trick, W. E., S. K. Fridkin, J. R. Edwards, R. A. Hajjeh, and R. P. Gaynes.** 2002. Secular trend of hospital-acquired candidemia among intensive care unit patients in the United States during 1989–1999. Clin. Infect. Dis. **35:**627–630.
35. **White, T. J., T. D. Bruns, S. B. Lee, and J. W. Taylor.** 1990. Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics, p. 315–322. *In* M. A. Innis, D. H. Gelfand, J. J. Sninsky, and T. J. White (ed.), PCR protocols: a guide to methods and applications. Academic Press, Inc., San Diego, CA.
36. **Wiederhold, N. P., and R. E. Lewis.** 2003. The echinocandin antifungals: an overview of the pharmacology, spectrum and clinical efficacy. Expert Opin. Investig. Drugs **12:**1313–1333.
37. **Wiederhold, N. P., L. K. Najvar, R. Bocanegra, D. Molina, M. Olivo, and J. R. Graybill.** 2007. In vivo efficacy of anidulafungin and caspofungin against *Candida glabrata* and association with in vitro potency in the presence of sera. Antimicrob. Agents Chemother. **51:**1616–1620.
38. **Wisplinghoff, H., T. Bischoff, S. M. Tallent, H. Seifert, R. P. Wenzel, and M. B. Edmond.** 2004. Nosocomial bloodstream infections in US hospitals: analysis of 24,179 cases from a prospective nationwide surveillance study. Clin. Infect. Dis. **39:**309–317.
39. **Zheng, W., J. S. Finkel, S. M. Landers, R. M. Long, and M. R. Culbertson.** 2008. Nonsense-mediated decay of ash1 nonsense transcripts in *Saccharomyces cerevisiae*. Genetics **180:**1391–1405.