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Mutations in the *fks1* Gene in *Candida albicans*, *C. tropicalis*, and *C. krusei* Correlate with Elevated Caspofungin MICs Uncovered in AM3 Medium Using the Method of the European Committee on Antibiotic Susceptibility Testing▽

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Mutations in two specific regions of the Fks1 subunit of 1,3-β-D-glucan synthase are known to confer decreased caspofungin susceptibility on *Candida* spp. Clinical isolates of *Candida* spp. (404 *Candida albicans*, 62 *C. tropicalis*, and 21 *C. krusei* isolates) sent to the French National Reference Center were prospectively screened for susceptibility to caspofungin in vitro by the broth microdilution reference method of the Antifungal Susceptibility Testing Subcommittee of the European Committee on Antibiotic Susceptibility Testing (AFST-EUCAST). Twenty-eight isolates (25 *C. albicans*, 2 *C. tropicalis*, and 1 *C. krusei* isolate) for which the caspofungin MIC was above the MIC that inhibited 90% of the isolates of the corresponding species (MIC_{90}) were subjected to molecular analysis in order to identify mutations in the *fks1* gene. Substitutions in the deduced protein sequence of Fks1 were found for 8 isolates, and 20 isolates had the wild-type sequence. Among the six *C. albicans* isolates harboring mutations, six patterns were observed involving amino acid changes at positions 641, 645, 649, and 1358. For *C. tropicalis*, one isolate showed an L644W mutation, and for one *C. krusei* isolate, two mutations, L658W and L701M, were found. Two media, RPMI medium and AM3, were tested for their abilities to distinguish between isolates with wild-type Fks1 and those with mutant Fks1. In RPMI medium, caspofungin MICs ranged from 0.25 to 2 μg/ml for wild-type isolates and from 1 to 8 μg/ml for mutant isolates. A sharper difference was observed in AM3: all wild-type isolates were inhibited by 0.25 μg/ml of caspofungin, while caspofungin MICs for all mutant isolates were ≥0.5 μg/ml. These data demonstrate that clinical isolates of *C. albicans*, *C. tropicalis*, and *C. krusei* with decreased susceptibility to caspofungin in vitro have diverse mutations in the *fks1* gene and that AM3 is potentially a better medium than RPMI for distinguishing between mutant and wild-type isolates using the AFST-EUCAST method.

Caspofungin is an echinocandin that inhibits 1,3-β-D-glucan synthesis in several fungal species involved in human infections, including *Candida* spp. and *Aspergillus* spp. (10). Caspofungin is used for the treatment of invasive candidiasis and aspergillosis as well as for oropharyngeal and esophageal candidiasis (9). The target of caspofungin is the enzyme 1,3-β-D-glucan synthase, encoded by one or several *fks* genes, depending on the species (14). It has been shown that in laboratory mutants as well as in some clinical isolates, mutations in the *fks1* gene resulting in amino acid changes in the protein were necessary and sufficient to confer reduced susceptibility to caspofungin (27, 28). These mutations, associated with reduced susceptibility to caspofungin, have been observed only within two "hot spot" (HS) regions of the Fks protein (located at amino acid positions 640 to 650 and 1345 to 1365 in *Candida albicans*) (22, 23, 27). Mutations in Fks2 have also been linked with echinocandin resistance in *Candida glabrata* (20).

A recent study failed to demonstrate a correlation between caspofungin MICs for *Candida* spp. isolates and clinical or microbiological outcomes for patients with esophageal or invasive candidiasis enrolled in four clinical trials (19). Nevertheless, cases of invasive and esophageal infections with isolates harboring decreased in vitro susceptibility to caspofungin have been reported (1, 7, 12, 15, 16, 20–24, 27, 32). In several instances, increased caspofungin MICs appeared during treatment with an echinocandin, and molecular studies of the pre- and posttreatment isolates demonstrated a genotypic identity suggesting the acquisition of resistance (1, 7, 12, 15, 16, 21, 22, 24, 27, 32). Elevated MICs were associated with mutations in the HS1 or HS2 region of the deduced Fks protein (1, 20, 22, 23, 27). A correlation between in vitro resistance and therapeutic failure was confirmed in vivo in an animal model of candidiasis (16, 21, 27). Furthermore, a comprehensive study including in vitro susceptibility, inhibition of glucan synthase activity, and response to antifungal therapy in an animal model of disseminated candidiasis demonstrated that mutations in the Fks1 protein are sufficient to confer reduced susceptibility to caspofungin (27).

If resistance correlates with therapeutic failure, it is of prime importance to be able to detect it routinely by an in vitro susceptibility testing method. However, the best methodolog-

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TABLE 1. Primer sequences used in this study

Species	Region	Primer	Sequence (5′–3′)	Amplicon size (bp)	Annealing temp (°C)
Candida albicans	HS1[a]	GSC1f	GAAATCGGCATATGCTGTGTC	450	50
		GSC1r	AATGAACGACCAATGGAGAAG		
	HS2	CAS2f	ACCACCAAGATTGGTGCTG	497	58
		CAS2r	TATCTAGCACCACCAACGG		
Candida krusei	HS1	CKS1f	ACTGCATCGTTTGCTCCTCT	500	50
		CKS1r	GAACATGATCAATTGCCAAC		
	HS2	CKS2f	CCGGTATGGGAGAACAAATG	474	58
		CKS2r	CACCACCAATGGAAACATCA		
Candida tropicalis	HS1	CTS1-1f	ATGGTTCAGTATAGGTGGATG	221	50
		CTS1-1r	AAGGAACGACCAATGGAGAAG		
	HS2	CTS1-2f	ACTACCAAGATTGGTGCTG	497	56
		CTS1-2r	TATCTAGCACCACCAACAG		

[a] HS1 primers were from Park et al. (27).

ical parameters to use for echinocandin susceptibility testing remain uncertain. It has been shown that antibiotic medium 3 (AM3) is superior to RPMI medium for testing caspofungin against *Candida* spp. (3). More recently, better discrimination between *Candida* spp. isolates of known high and low susceptibilities to caspofungin was achieved by using AM3 than by using RPMI medium (25).

The aims of the present study were (i) to identify isolates with mutations in the HS1 or HS2 region of the Fks protein among a large collection of clinical *Candida* spp. isolates and (ii) to test RPMI medium and AM3 for their abilities to distinguish between wild-type and Fks1 mutant isolates.

MATERIALS AND METHODS

Strains. *C. albicans*, *Candida tropicalis*, and *Candida krusei* isolates sent to the French National Reference Center for Mycoses and Antifungals from January 2005 to August 2007 were used for the present study. During that period, caspofungin MICs were prospectively determined by the method of the European Committee on Antibiotic Susceptibility Testing (EUCAST) in two different media, RPMI medium and AM3. Isolates with a caspofungin MIC above the MIC determined in the corresponding medium that inhibited 90% of the isolates of the same species (MIC_{90}) were subjected to molecular analysis in order to identify mutations in the Fks protein.

All isolates were subcultured on CHROMagar *Candida* medium (Becton Dickinson GmbH, Heidelberg, Germany) to ensure purity and viability. Isolates were identified at the species level by standard mycological procedures including the assimilation patterns obtained with commercialized ID32C strips (bioMérieux, Marcy-l'Etoile, France). For all *C. albicans* isolates, a specific PCR amplification (13) was performed to distinguish this species from *Candida dubliniensis*.

In vitro susceptibility testing. In vitro susceptibility was determined by a microdilution technique according to the guidelines of the reference procedure proposed by the Antifungal Susceptibility Testing Subcommittee of EUCAST (AFST-EUCAST) (31). A pure caspofungin powder of known potency (Merck and Co., Rahway, NJ) was used. Microplates were prepared by batch and stored frozen at −20°C. Briefly, testing was performed with a final inoculum size of 10^5 yeast cells/ml and a final concentration of echinocandin ranging from 0.015 to 8 μg/ml. Tests were performed in parallel in two media, RPMI 1640 (Sigma, Saint Quentin Fallavier, France) buffered to pH 7.0 with 0.165 M morpholinepropanesulfonic acid (MOPS) (Sigma) and AM3 (Difco, Becton Dickinson, Le Pont de Claix, France). Both media were supplemented with glucose to obtain a final concentration of 2% glucose. Two reference strains, *C. krusei* ATCC 6258 and *Candida parapsilosis* ATCC 22019, were included as quality controls. Microplates were incubated in ambient air in a humid environment for 24 h at 35°C. After shaking, fungal growth was determined by an automated microplate reader spectrophotometer (Multiscan RC-351; Labsystems Oy, Helsinki, Finland). The MIC end point was defined as a reduction of 50% or more in growth relative to that in the drug-free well. Microplates were also read after 48 h of incubation, and MICs with a 90% inhibition end point were determined at both times.

MIC data analysis. For calculation, the high off-scale MICs were converted to the next highest concentration and the low off-scale MICs were left unchanged. Distributions of MICs obtained in RPMI medium and AM3 were compared by a paired test (Wilcoxon). MICs for wild-type and mutant isolates were compared by a nonparametric test (Mann-Whitney). Statistical analyses were performed using GraphPad Prism, version 3.00 for Windows (GraphPad Software, San Diego, CA). Statistical significance was defined as a P value of ≤ 0.05.

Genomic DNA extraction. After 24 h of incubation at 27°C on Sabouraud agar plates, yeasts were discharged in 1 ml of distilled water in a microcentrifuge tube. Then DNA was extracted with the High Pure PCR template preparation kit (Roche Applied Science, Mannheim, Germany) according to the manufacturer's instructions.

PCR primer design, amplification, and sequence determination. Table 1 summarizes the nucleotide sequences of the primers used. Oligonucleotide primers were designed using Primer3, version 0.3.0 (http://frodo.wi.mit.edu/), and Oligonucleotide Properties Calculator software (http://www.basic.northwestern.edu/biotools/oligocalc.html). For *C. albicans*, primers described by Park and colleagues (27) were used for the amplification of the HS1 region of the gene coding for 1,3-β-glucan synthase (*fks1*). Specific primers were designed to amplify the sequence of the HS2 region based on the available sequence of the *fks1* gene (GenBank accession no. D88815). The nucleotide and protein sequences of the *fks1* genes of *C. tropicalis* and *C. krusei* were compared with the *C. albicans* sequences to determine the positions of the HS1 and HS2 regions. For *C. krusei*, primers were designed to amplify the sequences of the HS1 and HS2 regions by using the complete sequence of the *fks1* gene (accession no. EF426563). For *C. tropicalis*, the partial sequence of the *fks1* gene was used to design primers for the amplification of the HS1 and HS2 regions (http://www.broad.mit.edu/annotation/genome/candida_tropicalis/Home.html). Reaction volumes of 50 μl contained 3 μl of genomic DNA, 0.25 U of AmpliTaq Gold, 5 μl of 10× PCR buffer, 5 μl of 25 mM $MgCl_2$, 5 μl of 2.5 mM deoxynucleoside triphosphates (Roche), and 1.25 μl of 20 μM primers. The PCR products were amplified in a iCycler thermocycler (Bio-Rad, Marnes-La-Coquette, France) set up with a first cycle of denaturation for 10 min at 95°C, followed by 30 cycles of denaturation at 94°C for 30 s, annealing at the relevant temperature (Table 1) for 30 s, and elongation at 72°C for 30 s, with a final extension step of 10 min at 72°C. Both strands of purified amplified fragments were sequenced at the Genopole of the Pasteur Institute, on an ABI Prism 3700 DNA analyzer (Applied Biosystems, Courtaboeuf, France), with the same primers that were used in the PCR step. Sequences were edited with Chromas Pro software, version 1.33 (Technelysium Pty Ltd., Australia). Multiple sequence alignments were performed using Clustal W software, version 1.8. The sequences were translated with the standard genetic code (http://bioinformatics.org/sms/index.html). The resulting protein sequences were aligned with BioloMics software, version 7.2.5 (BioAware SA, Hannut, Belgium). Sequences of the two regions HS1 and HS2 for three reference strains (*C. albicans* B311 [ATCC 32354], *C. tropicalis* ATCC 750, and *C. krusei* ATCC 6258) were also amplified and sequenced. The positions of the nucleotides and amino acids for *C. albicans* and *C. krusei* were based on the complete *fks*1 genes (GenBank accession no. D88815 and EF426563, respectively). The positions of

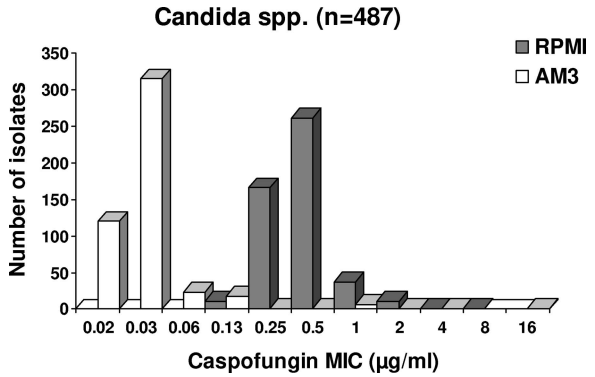

FIG. 1. Distribution of caspofungin MICs for 487 isolates of *Candida* spp., determined by the EUCAST reference technique in RPMI medium and AM3.

the nucleotides and amino acids for the *C. tropicalis* isolates were determined based on the coding sequence of the *fks1* gene and on the sequence of the Fks1 protein of *C. albicans*, respectively.

RESULTS

Distribution of caspofungin MICs. A total of 487 isolates (404 *C. albicans*, 62 *C. tropicalis*, and 21 *C. krusei* isolates) were analyzed. The caspofungin MIC distributions in RPMI medium and AM3 are shown in Fig. 1. Overall, MICs ranged from 0.125 to 8 μg/ml in RPMI medium and from 0.015 to 16 μg/ml in AM3 (Table 2). MICs were significantly ($P < 0.0001$) higher in RPMI medium (geometric mean MIC [GMIC], 0.42 μg/ml) than in AM3 (GMIC, 0.03 μg/ml). This difference between the two media was noted for all three species analyzed (Table 2), as well as for other species, such as *C. glabrata* and *Candida kefyr* (data not shown). In *C. albicans*, caspofungin MICs above the MIC_{90} were observed only in RPMI medium for 5 isolates, only in AM3 for 14 isolates, and in both media for 6 isolates. One *C. tropicalis* isolate had a MIC above the MIC_{90} only in RPMI medium, and one other isolate had a MIC above the MIC_{90} in both media. A caspofungin MIC above the MIC_{90} was detected (in both media) for one *C. krusei* isolate. These 28 isolates were recovered from blood ($n = 17$), oral cavities ($n = 4$), and other sites ($n = 7$) of 24 patients.

Molecular analysis of *fks1*. The nucleotide and corresponding deduced protein sequences of the HS1 and HS2 regions of 1,3-β-glucan synthase were determined for the 28 clinical isolates with MICs above the MIC_{90} (25 *C. albicans*, 2 *C. tropicalis*, and 1 *C. krusei* isolate) as well as for 1 reference strain for each species (Table 3). Overall, mutations conferring amino acid substitutions were found in 8 isolates (6 *C. albicans*, 1 *C. tropicalis*, and 1 *C. krusei* isolate), whereas no amino acid changes were found for 19 *C. albicans* isolates and 1 *C. tropicalis* isolate. Among the six *C. albicans* isolates harboring amino acid substitutions, six mutation patterns were observed involving amino acid changes at positions 641, 645, 649, and 1358. Homozygous mutations were observed for four isolates (in HS1 for three isolates and in HS2 for one isolate). Of the three isolates with homozygous mutations in HS1, one had T1922C and C1923T point mutations, resulting in the protein modification F641S; one had a T1933C mutation, resulting in an S645P substitution in the deduced protein sequence; and one had a C1934A mutation, leading to an S645Y substitution in the deduced protein sequence. In HS2, a T4072C mutation induced a W1358R amino acid change for one isolate. Heterozygous mutations were observed for two isolates. In one of these isolates, two amino acid changes in HS1 were associated (F641S and S645P, due to point mutations T1922Y and T1933Y, respectively); in the other, a heterozygous P649H change (due to a C1946M point mutation) in HS1 was associated with a heterozygous W1358R change (due to a T4072Y point mutation) in HS2. Additionally, for *C. albicans* isolates, two silent mutations in HS1 were noted. First, a mutation at position 1923 that was homozygous (C1923T) for one isolate and heterozygous (C1923Y) for two other isolates was observed. Second, mutation T1929W was observed in four isolates. In the HS2 region, there was only one isolate harboring a silent mutation (T4062W).

The *C. tropicalis* isolate showed a T-to-G point mutation in the HS1 region (equivalent to T1931G in *C. albicans fks1*), resulting in an L-to-W amino acid change (equivalent to position 644 in *C. albicans* Fks1p). Two missense mutations, T1973G and C2101A, were found in the HS1 region of the *C. krusei* isolate, resulting in the L658W and L701M amino acid changes, respectively. For *C. tropicalis* and *C. krusei*, there were no mutations in the HS2 region.

The eight resistant isolates were recovered from six patients and were isolated from blood culture ($n = 2$), urine ($n = 2$), bronchoalveolar lavage fluid ($n = 1$), and the oropharynx ($n = 3$). All eight isolates with missense mutations, either in HS1 or in HS2, exhibited caspofungin MICs of ≥1 μg/ml in RPMI medium (Table 3). Among the 20 isolates with a wild-type deduced Fks1 sequence, 5 *C. albicans* isolates and 1 *C. tropicalis* isolate had caspofungin MICs of 1 and 2 μg/ml in RPMI medium, respectively.

Comparison of RPMI medium and AM3 for detection of isolates with mutations. A comparison of caspofungin MIC distributions for isolates with a wild-type *fks1* gene and isolates with mutant *fks1* genes, according to the medium used, is shown in Fig. 2. Caspofungin MICs were significantly higher for mutant isolates than for wild-type isolates both in RPMI medium ($P < 0.0002$) and in AM3 ($P < 0.0001$). In RPMI medium, caspofungin MICs ranged from 0.25 to 2 μg/ml (GMIC, 0.54 μg/ml) for wild-type isolates and from 1 to 8 μg/ml (GMIC, 2.38 μg/ml) for mutant isolates. A sharper difference was observed in AM3, where all wild-type isolates were inhibited by 0.25 μg/ml of caspofungin (MIC range, 0.015 to 0.25 μg/ml; GMIC, 0.06 μg/ml) while all mutant isolates had

TABLE 2. Caspofungin MIC ranges and geometric mean MICs for 487 *Candida* sp. isolates tested in RPMI medium and AM3

Species (no. of isolates)	MIC (μg/ml)			
	In RPMI		In AM3	
	Range	Geometric mean	Range	Geometric mean
C. albicans (404)	0.125–4	0.38	0.015–16	0.03
C. krusei (21)	0.5–8	1.10	0.03–4	0.12
C. tropicalis (62)	0.25–2	0.60	0.015–1	0.03
All (487)	0.125–8	0.42	0.015–16	0.03

TABLE 3. Nucleotide and deduced protein sequences of the HS1 and HS2 regions of the *fks1* gene for 28 clinical isolates of *Candida* spp. and 3 reference strains

Isolate	Caspofungin MIC (μg/ml) at 24 h in:		HS1 region (amino acid positions 641–649)[a]		HS2 region (amino acid positions 1351–1358)[a]		Interpretation
	RPMI	AM3	Nucleotide sequence	Protein sequence	Nucleotide sequence	Protein sequence	
C. albicans							
B311	0.5	0.06	TTCTTGACTTTGTCTTTAAGAGATCCT	FLTLSLRDP	AATATTGCTCCTGCCGTTGATTGG	NIAPAVDW	Wild type
1, 2, 15, 25	0.25–1	0.03–0.25	TTCTTGAC**W**TTGTCTTTAAGAGATCCT	FLTLSLRDP	AATATTGCTCCTGCCGTTGATTGG	NIAPAVDW	Wild type
3	0.25	0.06	TTCTTGACTTTGTCTTTAAGAGATCCT	FLTLSLRDP	AATATTGCTCC**W**GCCGTTGATTGG	NIAPAVDW	Wild type
4–6, 8, 9, 11–13, 16, 17	0.5–1	0.03–0.125	TTCTTGACTTTGTCTTTAAGAGATCCT	FLTLSLRDP	AATATTGCTCCTGCCGTTGATTGG	NIAPAVDW	Wild type
7, 10	0.5	0.06–0.125	TT**Y**TTGACTTTGTCTTTAAGAGATCCT	FLTLSLRDP	AATATTGCTCCTGCCGTTGATTGG	NIAPAVDW	Wild type
14	1	0.03	TT**T**TTGACTTTGTCTTTAAGAGATCCT	FLTLSLRDP	AATATTGCTCCTGCCGTTGATTGG	NIAPAVDW	Wild type
18	1	0.015	TTCTTGACTTTGTCTTTAAGAGATCCT	FLTLSLRDP	AATATTGCTCCTGCCGTTGATTGG	NIAPAVDW	Wild type
19	1	0.5	TTCTTGACTTTGTCTTTAAGAGATC**M**T	FLTLSLRD**H**[c]	AATATTGCTCCTGCCGTTGAT**Y**GG	NIAPAVD**R**[c]	Mutant
20	2	2	T**Y**CTTGACTTTG**Y**CTTTAAGAGATCCT	**S**LTL**P**LRDP[c]	AATATTGCTCCTGCCGTTGATTGG	NIAPAVDW	Mutant
21	2	1	TTCTTGACTTTGT**A**TTTAAGAGATCCT	FLTL**Y**LRDP	AATATTGCTCCTGCCGTTGATTGG	NIAPAVDW	Mutant
22[b]	2	1	T**CT**TTGACTTTGTCTTTAAGAGATCCT	**S**LTLSLRDP	AATATTGCTCCTGCCGTTGATTGG	NIAPAVDW	Mutant
23	2	0.5	TTCTTGACTTTGTCTTTAAGAGATCCT	FLTLSLRDP	AATATTGCTCCTGCCGTTGAT**C**GG	NIAPAVD**R**	Mutant
24	4	16	TTCTTGACTTTG**C**CTTTAAGAGATCCT	FLTL**P**LRDP	AATATTGCTCCTGCCGTTGATTGG	NIAPAVDW	Mutant
C. tropicalis							
ATCC 750	0.5	0.06	TTCTTGACTTTGTCTTTAAGAGATCCA	FLTLSLRDP	AATCTTTCTCCAGCTGTTGATTGG	NLSPAVDW	Wild type
1	2	0.06	TTCTTGACTTTGTCTTTAAGAGATCCA	FLTLSLRDP	AATCTTTCTCCAGCTGTTGATTGG	NLSPAVDW	Wild type
2	2	1	TTCTTGACTT**G**GTCTTTAAGAGATCCA	FLT**W**SLRDP	AATCTTTCTCCAGCTGTTGATTGG	NLSPAVDW	Mutant
C. krusei							
ATCC 6258	1	0.06	TTCCTTATTTTGTCCATTAGAGATCCA (. . .)ACTGATCTG	FLILSIRDP (. . .)TDL	AATTTAGCTCCAGCAATTGATTGG	NLAPAIDW	Wild type
1	8	4	TTCCTTATTT**G**GTCCATTAGAGATCCA (. . .)ACTGAT**A**TG	FLI**W**SIRDP (. . .)TD**M**	AATTTAGCTCCAGCAATTGATTGG	NLAPAIDW	Mutant

[a] Positions for *C. albicans* and *C. tropicalis* (positions for *C. tropicalis* are defined as equivalent to those for *C. albicans*). The whole HS1 and HS2 regions were analyzed, but only the regions harboring mutations are shown (according to Park et al. [27], HS1 and HS2 are defined as the regions between positions 640 and 650 and positions 1345 and 1365, respectively). For *C. krusei*, the amino acid positions shown are 655 to 701 and 1358 to 1365 (based on the complete sequences of the *fks1* gene [accession no. EF426563]) for HS1 and HS2, respectively. Boldfaced nucleotides and amino acids indicate changes from the consensus sequence.

[b] The HS1 sequence of this isolate was published previously (1).

[c] The mutation(s) is heterozygous. In the corresponding nucleotide sequence, W represents A or T; Y represents T or C; M represents A or C.

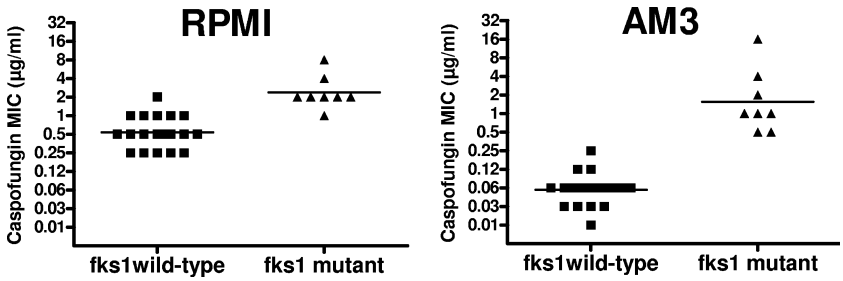

FIG. 2. Caspofungin susceptibilities of 8 *Candida* spp. isolates with mutations in the *fks1* gene and 20 wild-type isolates. MICs were determined by the EUCAST reference technique in RPMI medium (A) and AM3 (B). Horizontal lines represent GMICs.

caspofungin MICs of ≥0.5 μg/ml (MIC range, 0.5 to 16 μg/ml; GMIC, 1.54 μg/ml). MICs obtained either in RPMI medium or in AM3 by using a more stringent end point (90%) or an extended incubation time (48 h) did not distinguish better between resistant and susceptible isolates (data not shown).

DISCUSSION

A large collection of clinical isolates of *C. albicans*, *C. tropicalis*, and *C. krusei* was screened for caspofungin susceptibility by the AFST-EUCAST broth microdilution reference method. For the 28 isolates for which MICs were above the MIC_{90}, sequencing of the *fks1* gene coding for the 1,3-β-glucan synthase showed missense mutations in 8 isolates. These mutant isolates were recovered from different samples, including samples from deep and superficial body sites. Diverse mutations were found, including mutations that have never been reported previously. For *C. albicans*, mutations were found in HS1 at amino acid positions 641 (F641S), 645 (S645P or S645Y), and 649 (P649H) and in HS2 at position 1358 (W1358R). Most of the mutations described previously, both in clinical isolates and in laboratory mutants of *C. albicans* with reduced susceptibility to caspofungin, involved the serine residue at position 645 (2, 22, 23, 27). Another mutation involved phenylalanine at position 641 in three clinical isolates (1, 20) (one of these isolates is included in the present study) and in some laboratory mutants (2). Two additional mutations involving either leucine at position 644 in a laboratory mutant (2) or arginine at position 1361 in a clinical isolate (22) have been reported, associated with a substitution of Ser645. In a recent study, a large collection of 85 caspofungin-resistant laboratory mutants derived from two distinct parent strains were analyzed for *fks1* mutations, and substitutions were found at positions 645, 641, and 644 in 93, 6, and 1% of the mutants, respectively (2). The results of the present study and previous reports demonstrate that alteration of several amino acids in HS1 or HS2 can be associated with elevated caspofungin MICs for *C. albicans*, and this was observed with both the CLSI and the EUCAST methods. Here we also found an L-to-W mutation (equivalent to position 644 in *C. albicans* Fks1p) in *C. tropicalis*. To our knowledge, mutations in *fks1* associated with decreased in vitro susceptibility to caspofungin have never been reported for *C. tropicalis*. In the single *C. krusei* isolate with an elevated caspofungin MIC in our collection, two amino acid substitutions that had not been described to date, L658W and L701M, were found in HS1. Two *C. krusei* clinical isolates with mutations in *fks1* have been reported previously. In the first isolate, an R1361G mutation in HS2 was associated with a marked decrease in susceptibility to caspofungin in vitro (27). For the second isolate, no mutation was found at first (15), but a subsequent analysis showed a mutation in HS1 involving a phenylalanine residue (18). These results showed that, as in *C. albicans*, diverse mutations can be associated with reduced caspofungin susceptibility in *C. krusei*. It should be noted that although the new mutations reported in the present study were associated with elevated caspofungin MICs, the role of these mutations in the phenotype has to be confirmed by further analyses. In particular, it could be of interest to test the glucan synthase inhibition in vitro and to transform a susceptible strain with the mutant *fks1* gene. Moreover, mutant strains could be characterized in vivo in animal models to establish the important link between in vitro susceptibility and in vivo response to therapy. For the 20 wild-type isolates, only *fks1* has been sequenced, and the presence of mutations in *fks* homologue genes cannot be ruled out.

It is noteworthy that diploid organisms such as *C. albicans* and *C. krusei* (17) can harbor both homozygous and heterozygous mutations in *fks1* (1, 2, 18, 20, 27). Both heterozygous and homozygous laboratory mutant isolates showed high caspofungin MICs (27), but they differed by their abilities to inhibit glucan synthesis in vitro and by their responses to caspofungin treatment in a mouse model of candidiasis. Indeed, the dose of caspofungin that reduced the fungal burden in the kidneys by 90% was 0.07 to 0.14 mg/kg of body weight for mice infected with heterozygous mutants compared to 3.2 mg/kg for mice infected with the homozygous mutant (27). In the present study, we found heterozygous mutations for only two isolates, but for each one, two mutations at different locations were associated. The relationships between the homozygous/heterozygous state and the level of reduced caspofungin susceptibility in clinical isolates remain to be explored with a larger number of strains.

The best technique for testing the susceptibility of yeasts to caspofungin in vitro is still under scrutiny. Large surveillance studies of in vitro caspofungin susceptibility have been performed by the standardized broth microdilution techniques of the CLSI (26, 29) and ASFT-EUCAST (5, 6, 8) using RPMI medium as the test medium. These techniques have proven to be reproducible, and a good correlation between the results obtained by these two methodologies has been demonstrated (4). Nevertheless, results from a large multicenter study show better interlaboratory reproducibility for the CLSI method

(with readings taken at 24 h of incubation) than for the EUCAST technique (25). This study also suggests that AM3 could be a better medium than RPMI for distinguishing between *Candida* spp. with normal and reduced susceptibilities to caspofungin (25). The components of AM3 are not completely defined, and lot-to-lot variations have been reported, but interlaboratory variabilities for caspofungin susceptibility testing were similar for AM3 and RPMI medium when the CLSI technique was used (25). Because the influence of the culture medium has not been evaluated for the reference broth microdilution technique developed by EUCAST, we prospectively determined MICs by this technique in two media, RPMI medium and AM3. We first showed that the MICs were indeed lower in AM3 than in RPMI medium, as reported with the CLSI method (25). More importantly, we showed by testing wild-type clinical isolates and a set of well-characterized clinical isolates harboring diverse mutations in the *fks1* gene that the two media differ in their abilities to detect *Candida* spp. isolates with reduced caspofungin susceptibility. In both media, Fks1 mutant isolates had higher MICs than wild-type isolates, but some of the mutant isolates were not distinguishable from wild-type isolates when tested in RPMI medium. In contrast, in AM3, very good discrimination was obtained: 100% of mutant isolates had MICs higher than the highest MIC for a wild-type isolate. Although the present study was not designed to determine a breakpoint, it is interesting that a MIC of $\geq$0.5 μg/ml in AM3 characterized all mutant isolates. These results have to be confirmed with other species, other types of mutations, and even other echinocandin drugs. In a recent study, we demonstrated that the Etest, which has been shown to be useful for caspofungin susceptibility testing (30) and for the detection of two caspofungin-resistant clinical isolates of *C. albicans* in one patient (1), was able to distinguish between isolates with wild-type and mutant *fks1* genes (11).

In conclusion, we have demonstrated that (i) clinical isolates of *C. albicans*, *C. tropicalis*, and *C. krusei* with decreased in vitro susceptibilities to caspofungin have missense mutations in the HS1 or HS2 region of the *fks1* gene; (ii) these mutations are even more diverse than currently reported; and (iii) AM3 is potentially a better medium than RPMI for distinguishing between mutant and wild-type isolates by using the AFST-EUCAST method.

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