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Acquired Echinocandin Resistance in a *Candida krusei* Isolate Due to Modification of Glucan Synthase▽

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A *Candida krusei* strain from a patient with acute myelogenous leukemia that displayed reduced susceptibility to echinocandin drugs contained a heterozygous mutation, T2080K, in *FKS1*. The resulting Phe655→Cys substitution altered the sensitivity of glucan synthase to echinocandin drugs, consistent with a common mechanism for echinocandin resistance in *Candida* spp.

Clinical antifungal resistance in fungi has been best characterized with azole drugs and is associated with a variety of mechanisms including direct modification of the target enzyme, levels of the target enzyme, decreased drug accumulation due to efflux pump overexpression, or some combination of these mechanisms (15). Less is known about reduced echinocandin susceptibility (RES), but resistance in *Candida* spp. has been associated only with mutations in the Fks1p subunit of the target enzyme (1,3)-β-D-glucan synthase (4–6, 10). Hakki et al. recently reported a case of invasive and oropharyngeal candidiasis caused by a *Candida krusei* isolate that developed RES after caspofungin (CFG) therapy. The preliminary evaluation did not reveal any modification of the *FKS1* gene sequence in the isolate with reduced susceptibility (2). To determine if the RES phenotype in this strain could be due to a different mechanism, pre- and post-CFG treatment strains were further assessed for genetic and biochemical modifications of glucan synthase.

Echinocandin susceptibility testing with CFG, micafungin, and anidulafungin was performed in triplicate using the broth microdilution method of CLSI (formerly NCCLS) document M27-A2 (8) with modifications (10). *C. krusei* strains (2) Ck98 (pretreament) and Ck100 (posttreatment) were grown with vigorous shaking at 30°C to early-stationary phase in YPD broth (2% yeast extract, 4% Bacto peptone, 4% dextrose). Glucan synthase isolation and CFG titration were done as described previously (10). Inhibition curves and 50% inhibitory concentrations (IC_{50}s) were determined using a sigmoidal response (variable-slope) curve and a two-site competition fitting algorithm with GraphPad Prism, version 4.0, software (Prism Software, Irvine, CA). Genomic DNA, obtained twice from each of five separate colonies, was extracted from cells grown overnight in YPD broth medium with the Q-Biogene (Irvine, CA) FastDNA kit. PCR amplification was performed on an iCycler thermocycler (Bio-Rad Laboratories, Hercules, CA). The *FKS1* genes from both strains were PCR amplified using Sure-Pol DNA polymerase (Denville Scientific Inc., Metuchen, NJ). DNA sequencing of the 5.9-kb gene was performed with a CEQ dye terminator cycle sequencing Quick Start kit (Beckman Coulter, Fullerton, CA) according to the manufacturer's recommendations. Sequencing analyses were done with the

TABLE 1. Echinocandin susceptibilities of *C. krusei* clinical strains

C. krusei isolate	MIC (μg/ml)[a] of:		
	Caspofungin	Micafungin	Anidulafungin
Ck-98	0.25	0.25	0.25
Ck-100	8.0	4.0	4.0

[a] Geometric mean (three repetitions on three separate days).

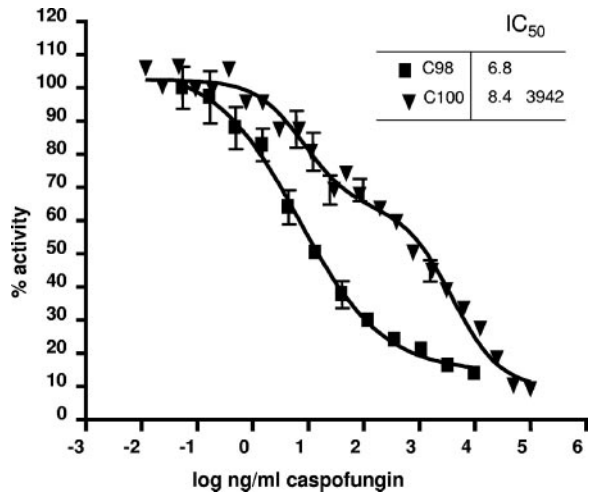

FIG. 1. CFG inhibition profiles for product-entrapped glucan synthases. Enzyme complexes were obtained from pretreatment (Ck-98) (squares) and posttreatment (Ck-100) (triangles) *C. krusei* strains. Inhibition curves and IC_{50}s (in nanograms per milliliter) were obtained using a sigmoidal response (variable-slope) curve (strain Ck-98) and a two-site competition fitting algorithm (strain Ck-100).

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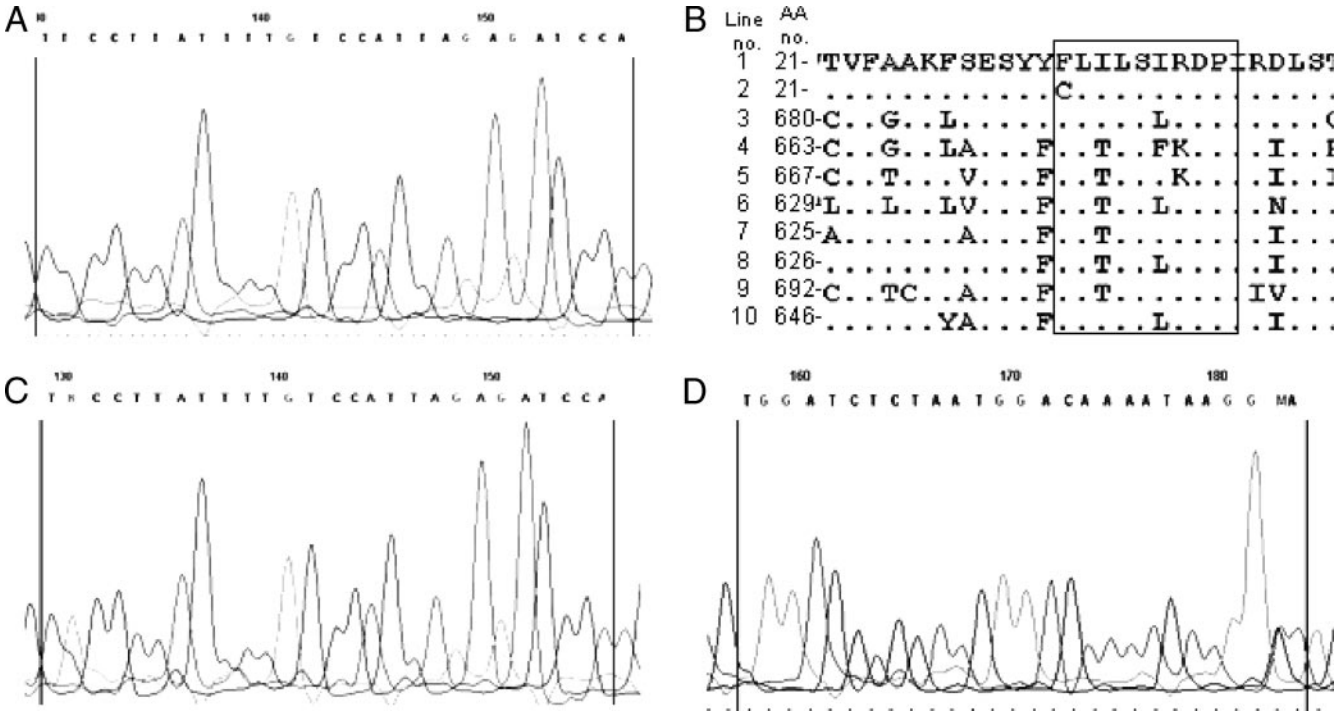

FIG. 2. DNA sequencing chromatograms and amino acid sequence alignment for *C. krusei* strains. (A) *C. krusei* strain Ck-98 Fks1p HS1 (forward primer). (B) Sequence alignments of the Fks1p HS1 region (boxed) in different fungal species. Line 1, *C. krusei* wild-type allele (GenBank accession no. AAY40291.1); line 2, *C. krusei* Ck-100 mutated allele; line 3, *Yarrowia lipolytica* (XP_504213); line 4, *Aspergillus fumigatus* (AAB58492); line 5, *Coccidioides immitis* (EAS36399); line 6, *C. albicans* (XP_721429); line 7, *Debaryomyces hansenii* (XP 457762); line 8, *Kluyveromyces lactis* (CAH02189); line 9, *Schizosaccharomyces pombe* (NP_588501); line 10, *Saccharomyces cerevisae* (AAZ22447). (C and D) *C. krusei* strain Ck-100 Fks1p HS1. (C) Forward primer; (D) reverse primer.

CEQ 8000 genetic analysis system software (Beckman Coulter, Fullerton, CA) and with the BioEdit sequence alignment editor (Ibis Therapeutics, Carlsbad, CA).

The MICs of echinocandin drugs for strain Ck-100 were 16- to 32-fold higher than those for the susceptible pretreatment strain, Ck-98 (Table 1), as previously reported (2). Moreover, the CFG MIC for Ck-100 exceeded the maximum range of 1 μg/ml reported for several large collections of clinical isolates using the CLSI M27-A2 method (9, 11–14). The reduced microbiological susceptibility of Ck-100 to CFG was confirmed in glucan synthase enzyme assays. A standard monophasic inhibition curve was obtained for Ck-98, yielding an IC_{50} of 6.8 ng/ml (Fig. 1). In contrast, biphasic inhibition was obtained for the product-entrapped enzyme from strain Ck-100, with IC_{50}s of 8.4 ng/ml and 3,942 ng/ml (Fig. 1). The presence of two distinct inflection points (two IC_{50}s) suggested a possible heterozygous genotype, as has been observed previously with *Candida albicans* (5, 6, 10). DNA sequence analysis of the entire *fks1* gene uncovered heterozygosity at a single nucleotide position, nucleotide T2080K, based on GenBank accession no. EF426563 (equivalent to nucleotide T2629 in *C. albicans* GenBank accession no. D88815). Both the wild-type and G-substituted *fks1* genes were observed in each of the five PCR products analyzed from strain Ck-100 (Fig. 2C and D). This nucleotide heterozygosity results in deduced amino acid heterogeneity—the wild type TTC codon encoding F and the modified TGC codon encoding C (Fig. 2B). None of the PCR products amplified from Ck-98 genomic DNA shared this nucleotide substitution (Fig. 2A). Failure to detect mutations in *FKS1* previously (2) likely results from the analysis of only one cloned allele. Since *C. krusei* is a diploid or aneuploid organism (3, 17), the cloning of one allele or another in a bacterial plasmid is a matter of chance.

The amino acid substitution at this position in *C. krusei* Fks1p is located in the highly conserved "hot spot 1" region of the protein (Fig. 2B) (10). Heterozygous amino acid changes in this region are associated with CFG resistance in both *Saccharomyces cerevisiae* laboratory strains and *C. albicans* clinical isolates (5, 6, 10). However, the only other reported *C. krusei* clinical isolate with RES had a predicted R1361G substitution in "hot spot 2" of Fks1p (10). Thus, clinical isolate Ck-100 is the first echinocandin-resistant *C. krusei* strain to be associated with a mutation in "hot spot 1."

The results obtained in this work highlight the need to monitor *C. krusei* infections treated with echinocandin drugs for the development of resistance. This is especially important because mortality from *C. krusei*, like that from other *Candida* spp., remains high (7, 16, 18) and treatment options are limited due to intrinsic resistance to fluconazole (1).

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