Molecular Confirmation of the Relationship between *Candida guilliermondii* Fks1p Naturally Occurring Amino Acid Substitutions and Its Intrinsic Reduced Echinocandin Susceptibility

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ABSTRACT *Candida guilliermondii* shows intrinsic reduced echinocandin susceptibility. It harbors two polymorphisms (L633M and T634A) in the Fks1p hot spot 1 region. Our objective was to confirm that the reduced echinocandin susceptibility of *C. guilliermondii* is due to those naturally occurring substitutions. We constructed a *Saccharomyces cerevisiae* mutant in which a region of the *FKS1* gene (including hot spot 1) was replaced with that from *C. guilliermondii*. The chimeric mutants showed 32-fold increases in echinocandin MIC values, confirming the hypothesis.

KEYWORDS *Candida guilliermondii*, *FKS* mutations, echinocandin resistance, molecular mechanism

Echinocandins are the treatment of choice for candidemia, since most *Candida* spp. are susceptible to these drugs (1–4). However, some highly prevalent *Candida* spp., such as species of the *Candida parapsilosis* complex and *Candida guilliermondii*, show natural reduced echinocandin susceptibility (RES) (5–7). Echinocandin drugs interact with the Fksp subunits of the β-1,3-glucan synthase complex, inhibiting the biosynthesis of the principal cell wall glucan. Clinical resistance was linked to hot spot mutations in Fksp (5). It was molecularly confirmed that the *C. parapsilosis sensu lato* RES phenotype is due to a naturally occurring substitution (P660A) in the hot spot 1 region of Fks1p (8). *C. guilliermondii* harbors two polymorphisms (L633M and T634A) in the same Fks1p region; it was assumed that the RES in this species is attributable to these amino acid substitutions, but the hypothesis was never confirmed (5).

The objective of this work was to verify that the intrinsic RES of *C. guilliermondii* is due to the naturally occurring amino acid changes in the hot spot 1 region of Fks1p. To reach this goal, we constructed a *Saccharomyces cerevisiae* strain with a hybrid *FKS1* gene. A region of the *FKS1* gene that includes hot spot 1 was replaced with that from *C. guilliermondii*. We then evaluated the echinocandin susceptibility.

S. cerevisiae BY4742 was used as the parental strain to obtain *S. cerevisiae* LMDM 537 (BY4742 *FKS1* deletant in which codons 453 to 649 were replaced with *URA3*, i.e., fks1Δ453–649::URA3) (8). *C. guilliermondii* ATCC 6260 (*Meyerozyma guilliermondii*) was used to obtain the 695-nucleotide (nt) region of the *FKS1* gene, which was fused to generate the chimeric *FKS1* gene (*S. cerevisiae-C. guilliermondii*). DNA was extracted by phenol-based extraction (9). Caspofungin (CSF) and anidulafungin (ANF) susceptibility tests were performed following CLSI guidelines (10–12), with modifications. For *S.*
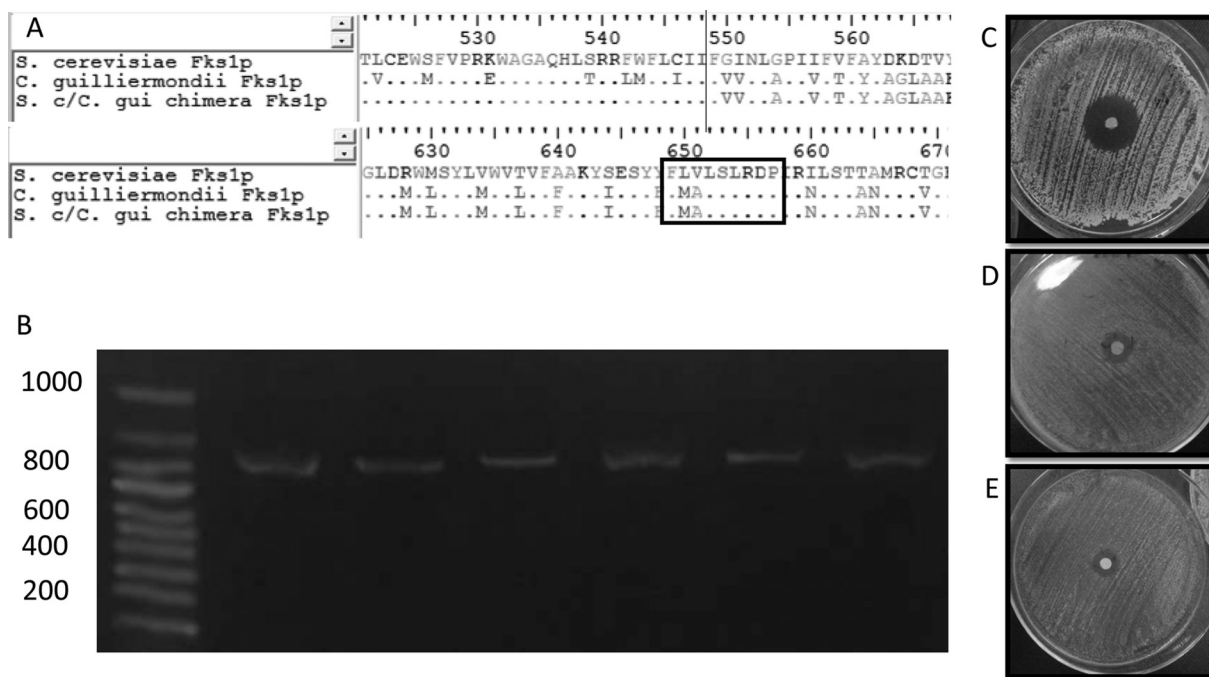
FIG 1 (A) Clustal alignment of the deduced Fks1p sequences of *S. cerevisiae* BY4742, *S. cerevisiae* harboring the chimeric *FKS1*, and *C. guilliermondii* ATCC 6260. Black lines show where the *C. guilliermondii FKS1* fragment was fused with the *S. cerevisiae* gene. The black box shows the FKS1 hot spot 1 region. (B) PCR confirmation of the *FKS1* reconstitution. Lane 1, markers; lanes 2 to 7, 872-nt PCR fragment of the chimeric *FKS1* gene obtained by using the FKS1-305F and Cg/h-R primers and DNA isolated from uracil-auxotrophic and CSF- and FK506-resistant *S. cerevisiae* transformants. (C, D, and E) Diffusion susceptibility testing using caspofungin disks, following CLSI guidelines (11), for *S. cerevisiae* BY4742 (C), *S. cerevisiae* harboring the chimeric *FKS1* (D), and *C. guilliermondii* ATCC 6260 (E).

cerevisiae susceptibility evaluations, RPMI 1640 broth and Mueller-Hinton 2% glucose-methyl blue agar were replaced by YPD broth (1% yeast extract, 2% peptone, 2% glucose) and YPD agar (YPD broth plus 1.5% agar), respectively. Also, the incubation temperature was changed to 30°C. These modifications were necessary since *S. cerevisiae* strains did not grow properly with the CLSI-proposed culture media and temperature (8).

A two-step method for PCR-based *FKS1* mutagenesis was employed to generate a *S. cerevisiae FKS1* chimeric mutant (8, 13, 14). Briefly, the first step consisted of partial deletion of the *FKS1* gene of *S. cerevisiae* BY4742 using an *URA3* cassette. The deletion included the Fks1p hot spot 1 region (amino acid residues 453 to 649). This disruption leads to FK506 (tacrolimus) and echinocandin hypersensitivity (8, 15). The transformation was performed and the deletion was confirmed using methodologies described previously (8, 16). The second step of the method was the replacement of the partially deleted *S. cerevisiae FKS1* gene (*fks1Δ453–649::URA3*) by a construction that included a 695-nt portion of the *C. guilliermondii FKS1* gene (nt 1591 to nt 2286 of the sequence reported in GenBank under accession number XM_001487741.1) surrounded by *S. cerevisiae FKS1* regions from nt 1123 to nt 1611 and from nt 2308 to nt 2548 (*S. cerevisiae FKS1* sequence reported in GenBank under accession number U12893.1). This construction was designed to yield, after homologous recombination, a functional *FKS1* gene encoding a chimeric Fks1p (*S. cerevisiae fks1Δ537–762::C. guilliermondii fks1 531–762*) (Fig. 1). The transformation vector was obtained by four PCRs, including amplification of the *C. guilliermondii FKS1* fragment (695 nt), the 488-nt portion of the 5′ region of *S. cerevisiae FKS1*, and the 240 nt of the 3′ fragment of *S. cerevisiae FKS1* using the primer pairs Fus5-FKS1-Sc-CguiF/Fus3-FKS1-Sc-CguiR, Fus5-FKS1-Sc-CguiR/ FKS1-375, and Fus3-FKS1-Sc-CguiF/LMDM85, respectively. The last PCR was designed to fuse the three fragments using FKS1-375 and LMDM85 primers (see Table 1 for primer details). PCRs were performed in a 25-μl volume, following the manufacturer's instructions for the Pegasus DNA polymerase (PBL, Buenos Aires, Argentina), in an Applied

TABLE 1 Primers used in this study

Primer	Target organism	Orientation (5′→3′)	Sequence (5′→3′)	Purpose	Reference
FKS1-453-URA3F	*S. cerevisiae*	Sense	AAAGAGACCCGTACTTGGTTACATTTGGTCACCAACTTCAGAGTG CACCATACCACAGCT	Generation of *S. cerevisiae FKS1*-deleted mutant	8
FKS1-649-URA3R	*S. cerevisiae*	Antisense	GTATTCACCTGTACACCTCATTGCAGTGGTGGACAAAATTGGTAT TTCACACCGCATAGG	Generation of *S. cerevisiae FKS1*-deleted mutant	8
URA3IR	*S. cerevisiae*	Antisense	TGCCTTTAGCGGCTTAACTG	Confirmation of *S. cerevisiae FKS1* deletion	8
FKS1-375	*S. cerevisiae*	Sense	GGTCGTTTTGTCAAGCGTGA	Confirmation of *S. cerevisiae FKS1* deletion, chimeric *FKS1* construction, and *S. cerevisiae FKS1* reconstruction	8
FKS1-707R	*S. cerevisiae*	Antisense	ATTTCCCAACAGAGAAAATGG	*S. cerevisiae FKS1* reconstruction	8
LMDM85	*S. cerevisiae*	Antisense	CGTAGTGAGGAGTCAATACTGTG	Chimeric *FKS1* construction	This study
Fus5-FKS1-Sc-CguiF	*S. cerevisiae* and *C. guilliermondii*	Sense	CGTAGATTCTGGTTTTTATGCATCATCTTCGTGGTTAACTTGGCCCC	Chimeric *FKS1* construction	This study
Fus5-FKS1-Sc-CguiR	*S. cerevisiae* and *C. guilliermondii*	Antisense	GGGGCCAAGTTAACCACGAAGATGATGCATAAAAACCAGAATCTACG	Chimeric *FKS1* construction	This study
Fus3-FKS1-Sc-CguiF	*S. cerevisiae* and *C. guilliermondii*	Sense	GTACAGAGAACATTTGTTGGCTATTGACCATGTACAAAAATTACTATATC	Chimeric *FKS1* construction	This study
Fus3-FKS1-Sc-CguiR	*S. cerevisiae* and *C. guilliermondii*	Antisense	GATATAGTAATTTTTGTACATGGTCAATAGCCAACAAATGTTCTCGTAC	Chimeric *FKS1* construction	This study
FKS1-305F	*S. cerevisiae*	Sense	CCCTGGAAAGAGTTCGTCATATC	Confirmation of *FKS1* chimeric reconstitution	This study
Cg/h-R	*C. guilliermondii*	Antisense	CAAACCACCCAAAGGCATAAC	Confirmation of *FKS1* chimeric reconstitution	This study

Biosystems thermocycler (Tecnolab-AB, Buenos Aires, Argentina). The construction was cloned into a PGEM-T Easy vector (Promega; Biodynamics, Buenos Aires, Argentina) to yield the plasmid LMDM-P113. *S. cerevisiae* LMDM 537 (BY4742 *fks1*Δ453– 649::*URA3*) was transformed with 1 μg of the LMDM-P113 plasmid, following the protocol (lithium acetate) described by Geitz et al. (17). Transformants were selected on FK506 plates and screened for the loss of *URA3* as described previously (8). The RES phenotype was screened for using YPD agar with 0.25 μg/ml CSF. Seven chimeric mutants showing the expected phenotype, i.e., uracil auxotrophic, FK506 resistant, and able to grow in the presence of 0.25 μg/ml CSF, were obtained. The *FKS1* reconstitution was assessed in all seven mutants by PCR using the primers FKS1-305F and Cg/h-R. These primers hybridize to the *S. cerevisiae FKS1* gene (around 900 nt upstream of the start codon) and *C. guilliermondii FKS* (near hot spot 1). The expected PCR band of 872 nt would be obtained only if the chimeric *FKS1* gene was present (Fig. 1). The *FKS1* reconstitution was confirmed by sequencing. A revertant control strain (LMDM 537R) was obtained by transforming a 1,000-bp PCR fragment into the *S. cerevisiae* LMDM 537 strain (17). This fragment was amplified using *S. cerevisiae* BY4742 DNA and the primers FKS1-375 and FKS1-707R (Table 1). The amplified fragment included the *S. cerevisiae FKS1* hot spot 1 region. Homologous recombination produced a strain with a functional *FKS1* that was FK506 resistant.

Parental and revertant *S. cerevisiae* strains (BY4742 and LMDM 537R, respectively) showed very low CSF and ANF MIC values (0.008 μg/ml), while the seven chimeric strains showed 32-fold higher MICs for both echinocandins (0.25 μg/ml). These values mimicked the *C. guilliermondii* ATCC 6260 RES phenotype (MICs of 0.25 μg/ml).

C. guilliermondii causes 1.4% of all *Candida* infections worldwide but is more prevalent in South America (>4%) (6, 18–20). Despite the RES phenotype, most of these infections are treated with echinocandins, since this yeast is also less susceptible to fluconazole than other *Candida* spp. (21). Most of these infections respond well to echinocandin therapy. However, *C. guilliermondii* breakthrough infections during caspofungin therapy have been reported (22). Under such circumstances, there is a concern that the use of echinocandins would contribute to an increased prevalence of this species.

It is well known that echinocandin clinical resistance is linked to *FKS* hot spot substitutions (5). The 32-fold increases in echinocandins MICs observed for the seven chimeric *S. cerevisiae* mutants support the assumption that L633M and T634A polymorphisms at *C. guilliermondii* Fks1p hot spot 1 are responsible for the observed RES in this species.

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