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Molecular Mechanism of Flucytosine Resistance in *Candida lusitaniae*: Contribution of the *FCY2*, *FCY1*, and *FUR1* Genes to 5-Fluorouracil and Fluconazole Cross-Resistance▽

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Inactivation of the *FCY2* (cytosine permease), *FCY1* (cytosine deaminase), and *FUR1* (uracil phosphoribosyltransferase) genes in *Candida lusitaniae* produced two patterns of resistance to flucytosine. Mutant *fur1* demonstrated resistance to 5-fluorouracil, whereas mutants *fcy1* and *fcy2* demonstrated fluconazole resistance in the presence of subinhibitory flucytosine concentrations.

Flucytosine (5FC) is one of the oldest antifungal agents. When this drug is taken up and converted to 5-fluorouracil (5FU) by fungal cells, it inhibits DNA replication and protein synthesis (11). However, it should be administered in combination with amphotericin B or azole antifungal agents, such as fluconazole (FLC), because the frequency of resistance development precludes its use as a single agent (10). In a previous work, we demonstrated that 5FC resistance in four *Candida lusitaniae* clinical isolates was due to a defect of purine-cytosine permease and that these isolates were specifically cross-resistant to FLC when both the antifungals 5FC and FLC were used in combination (8). More recently, we provided molecular evidence that inactivation of the *FCY2* gene in *C. lusitaniae* promotes cross-resistance to the antifungal combination 5FC-FLC (3). The goal of this study was to determine the precise contribution of the two other main genes possibly involved in 5FC resistance, *FCY1* and *FUR1*, to the 5FC-FLC cross-resistance phenotype.

Cloning and disruption of *FCY1*, *FUR1*, and *FCY2* genes. BLAST analysis of the *C. lusitaniae* database (http://www.broad.mit.edu/annotation/fungi/candida_lusitaniae/) allowed identification of a 507-bp gene encoding a predicted protein of 153 amino acids (16.8 kDa) that bore a significant identity with other cytosine deaminases from *Candida albicans* (69%), *Candida glabrata* (65%), and *Saccharomyces cerevisiae* (64%). The *C. lusitaniae FCY1* gene was located on supercontig 1.8 from positions 326868 to 326357 and contained a predicted intron located at nucleotides 56 to 105 from the ATG codon. In the same way, we identified one gene putatively encoding a uracil phosphoribosyltransferase (UPRTase) protein of 216 amino acid residues (24.3 kDa) that exhibited strong identity with the Fur1p proteins of *C. albicans* (90%), *C. glabrata* (75%), and *S. cerevisiae* (74%). The 651-bp intronless *C. lusitaniae FUR1* gene was located on supercontig 1.6 from positions 699508 to 700158. The complete *FCY1* and *FUR1* genes with their 5′ and 3′ UTR were isolated by PCR amplification and cloned into pGEM-T (Promega). Cloning of the *C. lusitaniae FCY2* gene (GenBank accession no. AY506668) has been described in a previous work (3).

Null mutants were constructed for the *FCY2*, *FCY1*, and *FUR1* genes by using an improved integrative transformation system based upon the "*URA3*-blaster" strategy. For that, the central part of the coding region of each cloned gene was deleted by digestion with adequate restriction enzymes and was replaced by the GUN fragment; this fragment consisted of the *C. lusitaniae URA3* gene flanked on both sides by a noncoding 327-bp repeat (fragment REP) obtained by amplification from the prokaryotic *NPTI* gene encoding neomycin phosphotransferase. The resulting disruption cassettes (FCY1-GUN, FCY2-GUN, FUR1-GUN) were excised from the cloning vector with restriction enzymes and were separately used to transform strain 6936 $ura3_{[D95V]}$ to prototrophy, as described previously (4). Correct targeting to each locus was verified by Southern analysis of the genomic DNA of Ura^+ transformants (results not shown). Gene replacement resulted in genotypes $ura3_{[D95V]}$, *fcy2*Δ::REP-*URA3*-REP (abbreviated *fcy2*Δ::*URA3*), $ura3_{[D95V]}$, *fcy1*Δ::REP-*URA3*-REP (abbreviated *fcy1*Δ::*URA3*), and $ura3_{[D95V]}$, *fur1*Δ::REP-*URA3*-REP (abbreviated *fur1*Δ::*URA3*).

Antifungal susceptibilities of the transformants. Testing of the susceptibilities of reference strain 6936 and the null mutants to 5FC, 5FU, FLC, and the association 5FC-FLC was performed (Table 1). Strain 6936 was susceptible to all antifungals tested and to the 5FC-FLC association. Null mutants were all resistant to 5FC, with MICs varying according to the strain genotype. Mutants *fcy2*Δ::*URA3* and *fcy1*Δ::*URA3* displayed the lowest MIC of 5FC (64 to 128 μg/ml), whereas mutant *fur1*Δ::*URA3* had the highest MIC (≥512 μg/ml). Only mutant *fur1*Δ::*URA3* was strongly resistant to 5FU (MIC, ≥512

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TABLE 1. Susceptibilities to 5FC, 5FU, and FLC of *C. lusitaniae* wild-type strain 6936, null mutants, and the revertant strains constructed for this study[a]

Strain or genotype	MIC (μg/ml)			Growth in RPMI containing 5FC-FLC[d]
	5FC	5FU	FLC	
6936	≤0.5	≤0.5	2.0	−
*fcy2*Δ::REP-*URA3*-REP[b]	64.0	≤0.5	2.0	+(4–16)
*fcy1*Δ::REP-*URA3*-REP[b]	128.0	≤0.5	2.0	+(8–32)
*fur1*Δ::REP-*URA3*-REP[b]	≥512.0	≥512.0	2.0	−
*fcy2*Δ::[*FCY2-URA3*]::REP[c]	≤0.5	≤0.5	2.0	−
*fcy1*Δ::[*FCY1-URA3*]::REP[c]	≤0.5	≤0.5	2.0	−

[a] Susceptibility testing was performed by microdilution assays with RPMI medium according to CLSI (formerly NCCLS) standards (7) and as described previously (3, 8) by routinely using the quality control strain *Candida krusei* ATCC 6258.

[b] Null mutants were derived from auxotrophic strain 6936 $ura3_{[D95V]}$.

[c] Engineered revertants were derived from complementation experiments.

[d] Testing for the occurrence of 5FC-FLC cross-resistance was performed by using a 5FC concentration gradient from 256 to 0.5 μg/ml in association with a constant concentration of 16 μg/ml FLC. Values in parentheses correspond to the ranges of 5FC concentrations (in μg/ml) that allowed the growth of the strains (+); −, no growth.

μg/ml), whereas mutants *fcy2*Δ::*URA3* and *fcy1*Δ::*URA3* were as susceptible as strain 6936. All the strains tested were susceptible to FLC. However, when 5FC and FLC (at 16 μg/ml, i.e., 8× MIC) were used in association, *fcy2*Δ::*URA3* and *fcy1*Δ::*URA3* developed cross-resistance to FLC, allowing up to 50% of the growth observed for the drug-free control (results not shown) over a range of 5FC subinhibitory concentrations that ranged from 4 to 32 μg/ml 5FC.

Complementation of *fcy2* and *fcy1* null mutant alleles. Ura$^-$ clones resistant to 5-fluoro-orotic acid (5FOA) were selected from the *fcy2*Δ::*URA3* and *fcy1*Δ::*URA3* mutants; and their genetic organization, i.e., loss of the *URA3* gene and of one of the flanking REP fragments, was confirmed by Southern blot analysis (data not shown). The genotypes $ura3_{[D95V]}$ *fcy2*Δ::REP (abbreviated *fcy2*Δ) and $ura3_{[D95V]}$ *fcy1*Δ::REP (abbreviated and *fcy1*Δ) were assigned to the 5FOA-resistant clones. Attempts to select a $ura3_{[D95V]}$ *fur1*Δ::REP mutant failed recurrently, even when UMP or uridine was used as supplementation. Complementation plasmids containing the *URA3* and *FCY2* genes or the *URA3* and *FCY1* genes were used to transform the *fcy2*Δ and *fcy1*Δ mutants to prototrophy, respectively. Southern blotting (not shown) was used to demonstrate the occurrence of the relevant genotypes $ura3_{[D95V]}$ *fcy2*Δ::[REP-*URA3-FCY2*] and $ura3_{[D95V]}$ *fcy1*Δ::[REP-*URA3-FCY1*]. The antifungal susceptibilities of these genetically engineered revertants were identical to that of susceptible reference strain 6936 (Table 1). We concluded that reintroduction of functional *FCY2* and *FCY1* alleles in 5FC-resistant *fcy2*Δ and *fcy1*Δ mutants, respectively, was sufficient to restore antifungal susceptibility.

Discussion and conclusion. Null mutants defective in the main enzymatic steps involved in the uptake and metabolism of 5FC were obtained in *C. lusitaniae* by using a "*URA3*-blaster" transformation system (1, 5) that we developed specifically for this *Candida* yeast species. This system allowed selection of *fcy2*Δ::*URA3*, *fcy1*Δ::*URA3*, and *fur1*Δ::*URA3* mutants in a first round of transformation experiments. Mutants *fcy2*Δ and *fcy1*Δ, which had lost the *URA3* marker, were then easily counterselected on 5FOA-containing medium and were used as recipient strains to successfully reintroduce functional *FCY2* and *FCY1* wild alleles. Nevertheless, a *fur1*Δ mutant could not be counterselected on 5FOA, probably because the combination of the *ura3* and *fur1*Δ mutations resulted in synthetic lethality, as has already been described in *S. cerevisiae* (6).

Testing for the susceptibilities of the null mutants to 5FC, 5FU, FLC, and the association 5FC-FLC showed that they were all resistant to 5FC and as susceptible to FLC as reference strain 6936, from which they were derived. Mutant *fur1*Δ was cross-resistant to 5FU, demonstrating that a single block in UPRTase is sufficient for total prevention of the synthesis of toxic fluorinated compounds in the fungal cell. However, the *fur1*Δ mutant did not exhibit the 5FC-FLC cross-resistant phenotype, indicating that the 5FU which accumulated in the mutant cells did not play any role in cross-resistance to FLC. On the other hand, mutants harboring a *fcy1*Δ or a *fcy2*Δ allele were resistant to 5FC, susceptible to 5FU, and 5FC-FLC cross-resistant when both antifungals were used in combination. This study demonstrates that the 5FC-FLC cross-resistance phenotype in *C. lusitaniae* was promoted not only by disruption of the *FCY2* gene encoding purine-cytosine permease, as described previously (3), but also by inactivation of the *FCY1* gene encoding cytosine deaminase. Both mutations result in the accumulation of 5FC, indicating that the molecular events that lead to cross-resistance to FLC are mediated by the fluorinated cytosine.

It is now possible to assign the mutations responsible for 5FC resistance into two functional groups according to their cross-resistance pattern in *C. lusitaniae*. Those affecting the *FUR1* gene can be responsible for a 5FC-5FU cross-resistance pattern and can confer a very high level of resistance (MICs, ≥512 μg/ml) to both drugs. Those affecting *FCY1* or *FCY2* confer a 5FC-FLC cross-resistance pattern and confer a lower level of resistance to 5FC (MICs, 64 to 128 μg/ml) either because in *fcy1* mutants 5FC can behave as an imperfect substrate of cytidine deaminase (2) or can be subjected to spontaneous nonenzymatic deamination (2) or because in *fcy2* mutants 5FC can enter the cell through low-affinity permeases, as recently reported in *S. cerevisiae* (9).

Nucleotide sequence accession numbers. The *FCY1* and *FUR1* sequences have been deposited in GenBank database under accession nos. DQ372926 and DQ372917, respectively.

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