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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

Short title: Resistance to flucytosine in *Candida lusitaniae*

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SUMMARY (44 words)

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

TEXT as a note (1252 words)

Flucytosine (5FC) is one of the oldest antifungal agents used. When uptaken and converted to 5-fluorouracil (5FU) by fungal cells, this drug 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 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 antifungals, 5FC and FLC, were used in combination (8). More recently, we provided molecular evidence that inactivation of the *FCY2* gene in *C. lusitaniae* promoted 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 significant identity with other cytosine deaminase from *C. albicans* (69%), *Candida glabrata* (65%) and *Saccharomyces cerevisiae* (64%). The *C. lusitaniae FCY1* gene was located on supercontig 1.8 from positions 326868-326357 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 an UPRTase protein of 216 amino acid residues (24.3 kDa) exhibiting strong identity with Fur1p 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 699508-700158. The

complete *FCY1* and *FUR1* genes with their 5' and 3' UTR were isolated by PCR amplification and cloned into pGEM-T (Promega). *FCY1* and *FUR1* sequences have been deposited in GenBank database (Accession No.: DQ372926 and DQ372917, respectively). Cloning of the *C. lusitaniae FCY2* gene (Accession No.: AY506668) has been described in a previous work (3).

Null mutants were constructed for the *FCY2*, *FCY1* and *FUR1* genes 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 non-coding 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 previously described (5). 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 susceptibility of the transformants. Susceptibility testing of the reference strain 6936 and of the null mutants was performed to 5FC, 5FU, FLC, and to the association 5FC+FLC (Table 1). Strain 6936 was susceptible to all antifungals tested and to the 5FC/FLC association. Null mutants were all resistant to 5FC with MIC values varying according to the strain genotypes. The mutants *fcy2*Δ::*URA3* and *fcy1*Δ::*URA3* displayed the lowest MIC of 5FC (64 to 128 μg/ml), whereas the mutant *fur1*Δ::*URA3* had the highest MIC (≥ 512 μg/ml). Only the mutant *fur1*Δ::*URA3* was strongly resistant

to 5FU (≥ 512 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. 8x 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 in the drug free control (results not shown), over a range of 5FC subinhibitory concentrations varying from 4 to 32 µg/ml 5FC.

Complementation of the *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 fragment, was confirmed by Southern blot analysis (data not shown). The genotypes *ura3*$_{[D95V]}$, *fcy2*Δ::REP and *ura3*$_{[D95V]}$, *fcy1*Δ::REP, abbreviated *fcy2*Δ and *fcy1*Δ, respectively, were assigned to the 5FOA-resistant clones. Attempts for selecting a *ura3*$_{[D95V]}$, *fur1*Δ::REP mutant failed recurrently, even when using uridine monophosphate or uridine as supplementation. Complementation plasmids containing *URA3* and *FCY2* genes or *URA3* and *FCY1* genes were used to transform to prototrophy the *fcy2*Δ and *fcy1*Δ mutants, respectively. Southern blot (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*]. Antifungal susceptibility of these genetically engineered revertants was identical to that of the 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 for the main enzymatic steps involved in uptake and metabolism of 5FC were obtained in *C. lusitaniae* using a "*URA3*-blaster" transformation system (1,6), 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, that had lost the *URA3* marker, were then easily counter-selected on a 5FOA-containing medium and used as recipient strains to successfully reintroduce functional *FCY2* and *FCY1* wild alleles. Nevertheless, a *fur1*Δ mutant could not be counter-selected on 5FOA probably because the combination of *ura3* and *fur1*Δ mutations resulted in synthetic lethality, as already described in *S. cerevisiae* (7).

Susceptibility testing to 5FC, 5FU, FLC and to the association 5FC-FLC, of the null mutants showed that they were all resistant to 5FC, and as susceptible to FLC as the 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 to totally prevent the synthesis of toxic fluorinated compounds in the fungal cell. However, the mutant *fur1*Δ did not exhibit the 5FC-FLC cross-resistant phenotype, indicating that 5FU which accumulated in the mutant cells, did not play any role in cross-resistance to FLC. On the other hand, mutants harbouring a *fcy1*Δ or *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 not only promoted by disruption of the *FCY2* gene encoding purine-cytosine permease, as it was previously described (3), but also by inactivation of the *FCY1* gene encoding cytosine deaminase. Both mutations result in 5FC accumulation, indicating that the molecular events leading 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 resistance level (≥ 512 µg/ml) to both drugs. Those affecting

157 *FCY1* or *FCY2* confer a 5FC-FLC cross-resistance pattern, and confer a lower
158 resistance level to 5FC (64-128 µg/ml), either because in *fcy1* mutants 5FC can behave
159 as an imperfect substrate of cytidine deaminase (2), or can be subjected to
160 spontaneous non-enzymatic deamination (2), or because in *fcy2* mutants 5FC can
161 enter the cell through low-affinity permeases, as recently reported in *S. cerevisiae* (9).
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TABLE and LEGENDS

Table 1. Susceptibility to 5FC, 5FU and FLC of *C. lusitaniae* wild-type strain 6936, of null mutants and of revertant strains constructed in this study

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

Susceptibility testing was performed by microdilution assays using RPMI medium according to CLSI standards (4) and as previously described (3,8), using routinely the quality control strain *Candida krusei* ATCC 6258.

[a] Null mutants were derived from the auxotrophic strain 6936 *ura3*$_{[D95V]}$.

[b] Engineered revertants were derived from complementation experiments.

[c] Testing the occurrence of 5FC/FLC cross-resistance was performed using a 5FC concentration gradient from 256 to 0.5 μg/ml associated with a constant concentration of 16 μg/ml FLC. Values between brackets correspond to ranges of 5FC concentrations (μg/ml) allowing growth of strains (+); - : no growth.