A novel multidrug efflux transporter gene of the major facilitator superfamily from *Candida albicans* (*FLU1*) conferring resistance to fluconazole

David Calabrese, Jacques Bille and Dominique Sanglard

Author for correspondence: Dominique Sanglard. Tel: +41 21 314 40 83. Fax: +41 21 314 40 60.
e-mail: Dominique.Sanglard@chuv.hospvd.ch

Institut de Microbiologie,
Centre Hospitalier
Universitaire Vaudois
(CHUV), Rue de Bugnon,
CH-1011 Lausanne,
Switzerland

Azole resistance in *Candida albicans* can be mediated by several resistance mechanisms. Among these, alterations of the azole target enzyme and the overexpression of multidrug efflux transporter genes are the most frequent. To identify additional putative azole resistance genes in *C. albicans*, a genomic library from this organism was screened for complementation of fluconazole hypersusceptibility in *Saccharomyces cerevisiae* YKKB-13 lacking the ABC (ATP-binding cassette) transporter gene *PDR5*. Among the *C. albicans* genes obtained, a new gene was isolated and named *FLU1* (fluconazole resistance). The deduced amino acid sequence of *FLU1* showed similarity to *CaMDR1* (formerly *BEN*), a member of the major facilitator superfamily of multidrug efflux transporters. The expression of *FLU1* in YKKB-13 mediated not only resistance to fluconazole but also to cycloheximide among the different drugs tested. The disruption of *FLU1* in *C. albicans* had only a slight effect on fluconazole susceptibility; however, it resulted in hypersusceptibility to mycophenolic acid, thus suggesting that this compound could be a substrate for the protein encoded by *FLU1*. Disruption of *FLU1* in a background of *C. albicans* mutants with deletions in several multidrug efflux transporter genes, including *CDR1*, *CDR2* and *CaMDR1*, resulted in enhanced susceptibility to several azole derivatives. *FLU1* expression did not vary significantly between several pairs of azole-susceptible and azole-resistant *C. albicans* clinical isolates. Therefore, *FLU1* seems not to be required for the development of azole resistance in clinical isolates.

Keywords: multidrug efflux transporters, azole antifungal agents, *Candida albicans*

INTRODUCTION

Candida albicans is the major cause of oropharyngeal candidiasis (OPC), which is a frequent opportunistic infection observed in HIV-positive patients (Odds *et al.*, 1990). OPC can occur in up to 90% of these individuals during their illness (Vanden Bossche *et al.*, 1994a). To treat infections caused by *C. albicans*, only a few antifungal agents are available and among them, fluco-

nazole, which is a compound belonging to the class of azole antifungal agents, is by far the most commonly used antifungal (Powderly, 1994). The target of azole antifungal agents in yeast is a cytochrome P-450. This enzyme is involved in the 14 α -demethylation of lanosterol which is an important step in the biosynthesis of ergosterol (Vanden Bossche *et al.*, 1994b).

The repeated use of fluconazole to treat OPC episodes has led to the appearance of clinical resistance which had been often correlated with *in vitro* resistance (Odds *et al.*, 1996; Rex *et al.*, 1995; Troillet *et al.*, 1993). It was demonstrated in a recent study investigating the mechanisms of resistance to fluconazole in *C. albicans* isolates that the majority of resistant *C. albicans* isolates failed to accumulate the levels of fluconazole measured

Abbreviations: MF, major facilitator; MFS, major facilitator superfamily; OPC, oropharyngeal candidiasis.

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in susceptible isolates. This phenomenon was due to an enhanced efflux of fluconazole. Multidrug efflux transporters of two different classes were up-regulated in *C. albicans* azole-resistant isolates, including ABC transporters and major facilitators (MFs), and were identified as possible mediators for this effect (Sanglard *et al.*, 1995). Each of the genes for these two transporters classes, *CDR1* (Prasad *et al.*, 1995) and *CaMDR1* (Fling *et al.*, 1991), were shown to be up-regulated in individual *C. albicans* isolates resistant to azole antifungal agents.

Additional mechanisms have been demonstrated to participate in azole resistance in *C. albicans* clinical isolates. These separate mechanisms involve alterations in the cellular target of azole antifungals (Sanglard *et al.*, 1998) or alterations in the ergosterol biosynthetic pathway (Marichal & Vanden Bossche, 1995). However, other mechanisms of azole resistance may be still found in clinical isolates. We therefore attempted the cloning of azole resistance genes putatively involved in the resistance of clinical isolates by a functional complementation strategy. The screening of a *C. albicans* genome library in a *Saccharomyces cerevisiae* mutant lacking the ABC transporter gene *PDR5* allowed the cloning of several azole resistance genes. Previously isolated genes (*CDR1* and *CaMDR1*) (Fling *et al.*, 1991; Prasad *et al.*, 1995) were found in this screening. A new ABC transporter gene, *CDR2*, was also isolated and was shown to be co-ordinately up-regulated with *CDR1* in several *C. albicans* azole-resistant clinical isolates (Sanglard *et al.*, 1997). Three additional genes were also cloned, among them *ERG11* (encoding the cytochrome P450 lanosterol 14 α -demethylase), *FLU1* and *FLU2*, the latter now referred to as *CAP1* and encoding a transcription factor (Alarco *et al.*, 1997; Sanglard *et al.*, 1997). Here we report the characterization of *FLU1* and show that it encodes a new multidrug efflux transporter with similarity to the MF transporters.

METHODS

Strains. *C. albicans* CAF4-2 (Δ *ura3::imm434*/ Δ *ura3::imm434*) and the parent CAF2-1 (Δ *ura3::imm434*/*URA3*) are derived from the wild-type strain SC5314 and were obtained from B. Fonzi (Fonzi & Irwin, 1993). The *S. cerevisiae* strain YKKB-13 (*MATa*, *ura3-52 lys2-801^{amber} ade2-101^{ochre} trp1 Δ 63 his3- Δ 200 leu2- Δ 1 Δ *pdr5::TRP1*) has been described by Bissinger & Kuchler (1994). The genotypes of other yeast strains are listed in Table 1. *Escherichia coli* DH5 α (Hanahan, 1985) was used as a host for plasmid construction and propagation. The clinical yeast strains used in this study originated from the Institute of Microbiology (Centre Hospitalier Universitaire Vaudois, Switzerland) and are listed in Table 2. The strains were isolated from AIDS patients with OPC and have been described in a previous study (Sanglard *et al.*, 1995).*

Media. *C. albicans* strains were grown in YEPD complex medium consisting of 2% glucose, 2% Bacto peptone (Difco) and 1% yeast extract (Difco). YEPD agar plates contained 2% agar (Difco) as a supplement. Yeast Nitrogen Base (YNB; Difco) contained 2% glucose and 2% agar (Difco) and was used as a selective medium after transformation of *C. albicans*. Agar plates containing 50 μ g 5-fluoroorotic acid (5-FOA) ml⁻¹

were made for the regeneration of the *ura3* genetic marker in YNB selective medium supplemented with 50 μ g uridine ml⁻¹.

Accumulation of [³H]fluconazole in *C. albicans* isolates. Fluconazole accumulation experiments in *C. albicans* were performed with ³H-labelled fluconazole (Amersham) as described previously (Sanglard *et al.*, 1995), except that a single 20 min incubation was used. Each [³H]fluconazole accumulation experiment was repeated twice.

Construction of plasmids. For the disruption of *FLU1* in *C. albicans*, a 2.7 kb *SphI*–*BglII* fragment from pDS255 (Sanglard *et al.*, 1997) was subcloned into pMTL21 to yield pDIS1. A blunt-ended 3.7 kb *Sall*–*BglII* fragment from pMB-7 (Fonzi & Irwin, 1993) containing the *hisG-URA3-hisG* disruption cassette was inserted in the single *SnaBI* site of pDIS1 to create pDIS2. The linear 6.4 kb *SphI*–*BglII* fragment from pDIS2 was used for *FLU1* disruption experiments.

Transformation of *C. albicans* and *S. cerevisiae*. *C. albicans* CAF4-2 and other related strains were transformed by a LiAc procedure developed by Sanglard *et al.* (1996). After transformation with linear DNA fragments the cells were plated in YNB selective medium and incubated for 2–3 d at 30 °C. *S. cerevisiae* YKKB-13 was also transformed with plasmids by the same method, except that incubation time on selective medium was 3–4 d.

Northern blotting and signal quantification. Total RNA from yeasts was extracted and electrophoresed following the method described by Sanglard *et al.* (1995). Transfer of RNA was performed by capillary action on GeneScreen Plus membranes (NEN). Membranes were pre-hybridized at 42 °C, with a buffer consisting of 50% formamide, 1% SDS, 4 $\times$ SSC (1 $\times$ SSC is 0.15 M sodium chloride, 0.015 M sodium citrate), 10% dextran sulfate and 100 μ g salmon sperm DNA ml⁻¹. DNA probes were labelled with [α -³²P]dATP by random priming (Feinberg & Vogelstein, 1984) and added to the hybridization solution overnight at 42 °C. Washing steps were at high stringency, identical to those recommended by the supplier (NEN). The *TEF3* mRNAs were analysed using a 0.7 kb *EcoRI*–*PstI* fragment from pDC1 as described by Hube *et al.* (1994). Probes were stripped off in sequential hybridizations by boiling membranes for 10 min in TE buffer with 0.1% SDS.

Southern blotting. Genomic DNA from *C. albicans* strains was isolated from 5 ml cultures grown overnight in YEPD medium. Cells were collected by centrifugation and washed twice in TE. Pellets were resuspended in 5 ml PRO-Buffer (1 M sorbitol, 25 mM EDTA, 20 mM Tris/HCl, pH 7.5) and 50 μ g 100T Zymolyase ml⁻¹ (Seikagaku) and 0.1% β -mercaptoethanol (Sigma) were added. The mixture was incubated at 37 °C until complete cell wall digestion occurred (up to 30 min). After centrifugation the cell pellets were slowly resuspended in 2 ml lysing solution (0.1 M EDTA, 0.8% SDS, 50 μ g ml⁻¹ proteinase K, 0.1 M Tris/HCl, pH 7.5) and were incubated on ice for 10 min. After centrifugation (10 min at 5500 r.p.m.), supernatants were transferred into new tubes and DNA was precipitated with 5 ml ethanol. DNA pellets were gently resuspended in 0.5 ml TE containing 100 μ g RNase A ml⁻¹ (Roche) and incubated at 37 °C for 15 min. DNA from each culture was then precipitated with 2-propanol, transferred into Eppendorf tubes, washed with 70% ethanol and finally resuspended in TE. DNA was digested by restriction enzymes and size-fractionated by 1% agarose gel electrophoresis. The digested DNA was vacuum-blotted on Gene Screen Plus membranes. Prehybridization and hybridization of the membrane with labelled DNA probe were performed at 42 °C in a solution containing 50% formamide, 5 $\times$ SSC, 1%

Table 1. Genotypes of yeast strains used in this study

Strain	Parental strain	Genotype	Reference
CAF4-2	CAF2-1	$\Delta ura3::imm434/\Delta ura3::imm434$	Fonzi & Irwin (1993)
DSY448	DSY447	$\Delta cdr1::hisG-URA3-hisG/\Delta cdr1::hisG$	Sanglard <i>et al.</i> (1996)
DSY465	DSY464	$\Delta camdr1::hisG-URA3-hisG/\Delta camdr1::hisG$	Sanglard <i>et al.</i> (1996)
DSY468	DSY467	$\Delta cdr1::hisG/\Delta cdr1::hisG$	Sanglard <i>et al.</i> (1996)
		$\Delta camdr1::hisG-URA3-hisG/\Delta camdr1::hisG$	
DSY653	CAF4-2	$\Delta cdr2::hisG-URA3-hisG/\Delta cdr2::hisG$	Sanglard <i>et al.</i> (1997)
DSY654	DSY653	$\Delta cdr2::hisG/\Delta cdr2::hisG$	Sanglard <i>et al.</i> (1997)
DCY1	CAF4-2	$\Delta flu1::hisG-URA3-hisG/FLU1$	This study
DCY2	DCY1	$\Delta flu1::hisG/FLU1$	This study
DCY3	DCY2	$\Delta flu1::hisG/\Delta flu1::hisG-URA3-hisG$	This study
DCY4	DSY465	$\Delta camdr1::hisG/\Delta camdr1::hisG$	This study
DCY5	DCY4	$\Delta camdr1::hisG/\Delta camdr1::hisG$	
		$\Delta flu1::hisG-URA3-hisG/FLU1$	
DCY6	DCY5	$\Delta camdr1::hisG/\Delta camdr1::hisG$	This study
		$\Delta flu1::hisG/FLU1$	This study
DCY7	DCY6	$\Delta camdr1::hisG/\Delta camdr1::hisG$	
		$\Delta flu1::hisG/\Delta flu1::hisG-URA3-hisG$	
DCY8	DSY468	$\Delta cdr1::hisG/\Delta cdr1::hisG$	This study
		$\Delta camdr1::hisG/\Delta camdr1::hisG$	
DCY9	DCY8	$\Delta cdr1::hisG/\Delta cdr1::hisG$	
		$\Delta camdr1::hisG/\Delta camdr1::hisG$	This study
		$\Delta flu1::hisG-URA3-hisG/FLU1$	
DCY10	DCY9	$\Delta cdr1::hisG/\Delta cdr1::hisG$	
		$\Delta camdr1::hisG/\Delta camdr1::hisG$	This study
		$\Delta flu1::hisG/FLU1$	
DCY11	DCY10	$\Delta cdr1::hisG/\Delta cdr1::hisG$	
		$\Delta camdr1::hisG/\Delta camdr1::hisG$	This study
		$\Delta flu1::hisG/\Delta flu1::hisG-URA3-hisG$	
DCY12	DSY448	$\Delta cdr1::hisG/\Delta cdr1::hisG$	
		$\Delta flu1::hisG-URA3-hisG/FLU1$	This study
DCY13	DCY12	$\Delta cdr1::hisG/\Delta cdr1::hisG$	
		$\Delta flu1::hisG/FLU1$	
DCY14	DCY13	$\Delta cdr1::hisG/\Delta cdr1::hisG$	This study
		$\Delta flu1::hisG/\Delta flu1::hisG-URA3-hisG$	This study
DCY15	DSY653	$\Delta cdr2::hisG/\Delta cdr2::hisG$	
		$\Delta flu1::hisG-URA3-hisG/FLU1$	
DCY16	DCY15	$\Delta cdr2::hisG/\Delta cdr2::hisG$	This study
		$\Delta flu1::hisG/FLU1$	This study
DCY17	DCY16	$\Delta cdr2::hisG/\Delta cdr2::hisG$	
		$\Delta flu1::hisG/\Delta flu1::hisG-URA3-hisG$	
DCY18	DCY17	$\Delta cdr1::hisG/\Delta cdr1::hisG$	This study
		$\Delta cdr2::hisG/\Delta cdr2::hisG$	
		$\Delta flu1::hisG-URA3-hisG/FLU1$	
DCY19	DCY18	$\Delta cdr1::hisG/\Delta cdr1::hisG$	This study
		$\Delta cdr2::hisG/\Delta cdr2::hisG$	
		$\Delta flu1::hisG/FLU1$	
DCY20	DCY19	$\Delta cdr1::hisG/\Delta cdr1::hisG$	This study
		$\Delta cdr2::hisG/\Delta cdr2::hisG$	
		$\Delta flu1::hisG/\Delta flu1::hisG-URA3-hisG$	

SDS and 100 µg denatured salmon sperm DNA ml⁻¹. The probe was prepared by random priming as described above. The membrane was washed as recommended by the manufacturer and exposed at -70 °C on a Fuji X-ray film with an intensifying screen.

PCR amplifications. PCR buffers and *Taq* polymerase were from Boehringer Mannheim. Briefly, PCR was carried out in a Thermal Cycler 480 (Perkin Elmer) with a first cycle of denaturation for 4 min at 94 °C followed by 30 cycles of annealing at 54 °C for 2 min, elongation at 72 °C for 2 min

Table 2. Azole susceptibility of sequential *C. albicans* strains isolated from six HIV-positive patients with OPC

MICs were determined by a microdilution method as described previously (Sanglard *et al.*, 1995).

Patient no.	<i>C. albicans</i> isolate	MIC ($\mu\text{g ml}^{-1}$)			Reference
		Fluconazole	Ketoconazole	Itraconazole	
I	C27	1	0.015	0.031	Sanglard <i>et al.</i> (1995)
	C40	128	2	1	Sanglard <i>et al.</i> (1995)
II	C33	0.25	0.015	0.031	Sanglard <i>et al.</i> (1995)
	C26	> 128	4	> 2	Sanglard <i>et al.</i> (1995)
III	C43	0.25	0.015	0.031	Sanglard <i>et al.</i> (1995)
	C56	128	4	0.062	Sanglard <i>et al.</i> (1995)
IV	26	0.25	0.031	0.031	This study
	91	16	0.031	0.062	This study
V	70	1	0.002	0.062	This study
	74	16	0.125	0.5	This study
VI	741	0.25	0.015	0.015	This study
	742	16	0.062	0.25	This study

and denaturation at 94 °C for 30 s. PCR was completed by a final elongation step at 72 °C for 10 min. Primers for PCR are described in the legend to Fig. 4. Yeast DNA templates for PCR were prepared from overnight cultures by mechanical breakage with glass beads as described previously (Sanglard *et al.*, 1996).

Sequencing. Sequencing reactions were performed by standard protocols using an AutoRead kit (Pharmacia Amersham). The reactions were analysed on an ALF automated station (Pharmacia). Sequences were obtained by primer elongation using custom primers (Microsynth).

RESULTS

Isolation and sequence analysis of *FLU1*

Since the *S. cerevisiae* mutant YKKB-13 lacking the ABC transporter Pdr5p was hypersusceptible to azole antifungal agents (Sanglard *et al.*, 1995), this phenotype was used for complementing azole hypersusceptibility with a *C. albicans* genomic library. As reported in a previous study, six different plasmid classes were isolated (Sanglard *et al.*, 1995). Two classes contained the previously cloned genes *CDR1* (Prasad *et al.*, 1995) and *CaMDR1* (Fling *et al.*, 1991), and a third class contained the *CDR2* gene (Sanglard *et al.*, 1997). The fourth and fifth classes were represented by plasmids pDS257 and pDS270, which contained *CAP1* (Sanglard *et al.*, 1997) and *ERG11* (D. Sanglard, unpublished data), respectively. Plasmid pDS255 belonged to the latter class and contained a gene different from those already documented. pDS255 conferred resistance to fluconazole, cycloheximide, terbinafine, sulfomethuron, cerulenin and brefeldin A in *S. cerevisiae* YKKB-13 (Sanglard *et al.*, 1997).

The *C. albicans* DNA insert of pDS255 was subcloned into different fragments. As summarized in Fig. 1, a

minimal fragment of 4200 bp in pDS255-7 was still able to confer resistance to fluconazole and cycloheximide, but not to other azole derivatives such as itraconazole and ketoconazole. The nucleotide sequence of this fragment was determined and an uninterrupted ORF of 1833 bp, starting from the most upstream ATG codon, was detected. The gene encoding this ORF was named *FLU1* (fluconazole resistance). The 5' flanking region starting from this ATG codon displayed the typical structural organization of yeast promoters: an A at position -3 and a TATA box consisting of two overlapping TATA consensus sequences (TATATA and TATAAA) at position -127 (Chen & Struhl, 1988). A + T-rich regions (80% between -236 and -1, and 95% in a 28 bp tract between -109 and -82) were also observed and could serve as upstream promoter elements for the constitutive expression of *FLU1*, as suggested by Struhl *et al.* (1985). Chromosomal mapping of *FLU1* revealed its presence on chromosome 7 of the *C. albicans* genome. *FLU1* is situated on the published physical map in a location hybridizing to fosmid 11H9 (Chibana *et al.*, 1998).

The *FLU1* ORF encoded a protein with a calculated molecular mass of 67.6 kDa. A BLAST search of this ORF with available databases revealed most similarity with members of the MF superfamily (MFS). In particular, Flu1p exhibited 31.0% identity and 58.6% similarity to another MFS member, *C. albicans* CaMdr1p. Alignment of the two amino acid sequences showed that the N-terminal portion of Flu1p was very divergent, not only from CaMdr1p, but also from other MFS proteins (Nelissen *et al.*, 1995). The *FLU1* sequence revealed the highest similarity with YLL028w of *S. cerevisiae* (data not shown). The *FLU1* amino acid sequence displays the structure and organization of a protein with multiple transmembrane domains. As in the case of CaMdr1p and YLL028wp, Flu1p is composed of two homologous

Fig. 1. Complementation of drug susceptibility in *S. cerevisiae* YKKB-13 with *FLU1*. (a) YKKB-13 was transformed with pDS255 or derived plasmids containing subcloned DNA segments and with the parent plasmid YEp24 (control). Each transformant was grown in minimal medium with required amino acids. Five microlitres of a cell suspension at a concentration of 2×10^7 cells ml⁻¹ was spotted onto YEPD medium containing different drugs as indicated (concentrations in parentheses are given in µg ml⁻¹). (b) Maps of plasmids complementing (+) or failing to complement (–) fluconazole hypersusceptibility of YKKB-13. All plasmids were derived from pDS255. The arrow indicates length and direction of the *FLU1* ORF and the sequenced region is indicated by the dashed line. The hatched box represents the YEp24 vector backbone. [Ba/Sa] represents the sites of genomic DNA insertion at the *Sau3A* site of YEp24. B, *BglII*; N, *NcoI*; Nh, *NheI*; P, *PvuII*; S, *SphI*; Sa, *SaI*; X, *XbaI*.

halves, each containing six transmembrane spans. This structural organization is a typical characteristic of MFS efflux transporters.

Expression of *FLU1* in *C. albicans* clinical isolates

Several matched pairs of *C. albicans* clinical isolates were selected to address the possible expression of *FLU1* in *C. albicans*. Six isolates were taken from a previously published study (Sanglard *et al.*, 1995) and six additional isolates were from separate patients. Each pair contained an azole-susceptible and an azole-resistant isolate. The susceptibility to azole derivatives of these 12 isolates is given in Table 2. These clinical strains were sequential isolates from a group of HIV-positive patients and were characterized by different genotyping methods (Boerlin *et al.*, 1996). Each of these isolates was chosen

on the basis of their already known azole resistance mechanisms. Total RNA from these isolates was extracted and subjected to Northern blotting analysis with labelled probes corresponding to the multidrug efflux transporters *FLU1*, *CaMDR1*, *CDR1* and *CDR2*. The expression of these multidrug efflux transporter genes is presented in Fig. 2. The azole-resistant isolates from three patients (II, III and V) showed up-regulation of *CDR1*, correlating well with a decrease in azole susceptibility. The expression of *CDR2* mirrored the increased expression of *CDR1* in agreement with previously published results (Sanglard *et al.*, 1997). The azole-resistant isolates from patients I, IV and VI showed up-regulation of *CaMDR1* (Sanglard *et al.*, 1995). In contrast, *FLU1* expression did not parallel azole resistance as illustrated in Fig. 2(b). *FLU1* expression was higher in the azole-resistant isolate of one

Fig. 3. Schematic representation of the disruption of the *FLU1* gene before (a) and after (b) regeneration of the *ura3* marker by selection with 5-FOA. Maps of the wild-type allele are shown with the corresponding disrupted alleles. The location of the primers P1 (5'-TTATCGAGAAGAGCCACCAG-3') and P2 (5'-AAATATGAAGTCCATCTCC-3') used in PCR for the monitoring of disruption (see Fig. 4) are indicated with black arrows. B, *Bgl*II; S, *Sph*I; Sa, *Sal*I; Sn, *Sna*BI. The open arrows indicate the position of the *FLU1* ORF.

Disruption of *FLU1* in *C. albicans*

To investigate the effect of Flu1p in *C. albicans*, the gene encoding this protein was deleted by targeted gene disruption by the method described by Fonzi & Irwin (1993). Strain CAF4-2, as well as strains with different genetic backgrounds where other multidrug transporters genes (*CDR1*, *CDR2* and *CaMDR1*) were deleted (Table 1) were utilized as recipients for transformation with a linear *FLU1* fragment interrupted by the *hisG-URA3-hisG* cassette (see Fig. 3a). PCR was applied to screen Ura⁺ transformants for the correct localization of the disruption of the first *FLU1* allele. Briefly, this PCR approach consisted of using a pair of primers, one specific for *FLU1* and the other specific for the *hisG* gene contained in the disruption cassette (Fig. 3). This PCR allowed control of not only the insertion of the disruption cassette, but also its correct localization at one of the *FLU1* alleles. Fig. 4 shows the results of the PCR analysis. Each $\Delta flu1/FLU1$ heterozygote analysed after regeneration of the *ura3* genetic marker (DCY2,

Fig. 4. PCR monitoring of the disruption of the *FLU1* gene in CAF4-2 and in five deletion mutants (DSY465, DSY448, DSY468, DSY653 and DSY654). The sequences of the primers used, P1 and P2, are given in the legend to Fig. 3. The expected product sizes for amplification with primers P1 and P2 are 0.68 kb for a wild-type allele, 4.7 kb for the $\Delta flu1::hisG$ -*URA3*-*hisG* allele and 1.84 kb for the disrupted allele after 5-FOA treatment ($\Delta flu1::hisG$). The origin of genomic DNA from each strain is indicated. The products of PCR reactions were separated on an agarose gel and were from cells after disruption of the first and second *FLU1* alleles. Two bands, corresponding to the *FLU1* wild-type and to the disrupted alleles, are observed in heterozygous mutants (*FLU1*/ $\Delta flu1::hisG$). The 4.7 kb band, which was expected for the $\Delta flu1::hisG$ -*URA3*-*hisG* allele was not observed, probably due to the limit of extension of the *Taq* polymerase used in PCR. Molecular mass standards are indicated on the left. The PCR products from the wild-type strain SC5314 and from DNA-free reagents (–) were loaded on the right.

Fig. 5. Verification of *FLU1* disruption by Southern analysis. Samples (2 µg) of genomic DNA from *C. albicans* wild-type SC5314, heterozygous $\Delta flu1$ mutants (DCY2, DCY6, DCY10, DCY13, DCY16 and DCY19) and homozygous $\Delta flu1$ mutants (DCY3, DCY7, DCY11, DCY14, DCY17 and DCY20) were digested with *SpeI*, separated by agarose gel electrophoresis and transferred onto a GeneScreen Plus membrane. The membrane was probed with a labelled *FLU1* fragment as described in the legend to Fig. 2. The sizes of the *SpeI* fragments hybridizing with the *FLU1* probe were predicted to be 4.6 kb for the wild-type allele, 5.7 kb for the $\Delta flu1::hisG$ allele after 5-FOA regeneration and 8.2 kb for the $\Delta flu1::hisG$ -*URA3*-*hisG* allele. These sizes correspond approximately to those detected by the *FLU1* probe, as shown on the right. Molecular mass standards are indicated on the left.

DCY6, DCY10, DCY13, DCY16 and DCY19) still contained the wild-type *FLU1* allele amplified as a 0.68 kb fragment and the 1.84 kb fragment expected from the $\Delta flu1::hisG$ disrupted allele. These *C. albicans* strains, heterozygous for the first *FLU1* disrupted allele, were further utilized for the disruption of the second remaining wild-type allele. No signal corresponding to the amplified wild-type *FLU1* allele could be recovered

from the generated homozygous mutants (DCY3, DCY7, DCY11, DCY14, DCY17 and DCY20). A complementary verification of *FLU1* disruption in heterozygous and homozygous strains was monitored by Southern blotting (Fig. 5). Fragments of expected sizes were detected in this analysis and no wild-type *FLU1* fragment could be observed in all homozygous deletion mutants. To verify if residual expression of *FLU1* could

Fig. 6. Northern blot of total RNA from *FLU1/FLU1* parental strains (CAF4-2, DSY465, DSY448, DSY468, DSY653 and DSY654) and from $\Delta flu1/\Delta flu1$ homozygous mutant strains. Total RNA was extracted as described in Methods. Transfer and sequential hybridizations were performed as described in the legend to Fig. 2 with labelled *FLU1* and *TEF3* DNA fragments as probes.

still be monitored in the final homozygotes, a Northern blot was performed with total RNA extracted from these strains. As shown in Fig. 6, *FLU1* mRNA was detectable in the parent strain CAF4-2, as well as in multidrug transporter mutants DSY448, DSY465, DSY468, DSY653 and DSY654. However, no *FLU1* mRNA could be detected in the homozygous mutants DCY3, DCY7, DCY14, DCY11, DCY17 and DCY20, thus demonstrating that disruption of *FLU1* is associated with loss of expression.

Drug susceptibility assays

Cell viability was not altered when both *FLU1* alleles were disrupted in CAF4-2 and other multidrug transporter mutants. Growth and morphological aspects of the $\Delta flu1/\Delta flu1$ mutants were apparently not affected. The usefulness of multidrug transporter mutants in addressing the function of multidrug transporters and assigning unrelated compounds as possible substrates of these proteins in *C. albicans* was reported previously (Sanglard *et al.*, 1996, 1997). If a defined inhibitor reduces the growth of a mutant compared to the wild-type, it suggests that this substance accumulates in the intracellular cell compartments, due to the absence of an efflux transporter specific for this substance. The difference in growth between the CAF2-1 wild-type strain and DSY448 ($\Delta cdr1/\Delta cdr1$) on plates containing fluconazole illustrates this effect and demonstrates that fluconazole is a substrate for Cdr1p (Fig. 7). Comparisons of growth between CAF2-1 and six different strains in which *FLU1* was disrupted (DCY3, DCY7, DCY11, DCY14, DCY17 and DCY20) showed reduced growth on YEPD containing mycophenolic acid (Fig. 7) and thus indicates that mycophenolic acid is probably a substrate for Flu1p. With the exception of mycophenolic acid, where the increased susceptibility of the $\Delta flu1/\Delta flu1$ mutants was very noticeable, some smaller differences in growth were also seen when other compounds were added to the YEPD medium. Disruption of *FLU1* in CAF4-2 resulted in increased susceptibility to the three azoles fluconazole, ketoconazole and itraconazole (Fig. 7). Susceptibility to the different agents tested was dependent on the different genetic backgrounds. As shown previously by Sanglard *et al.* (1996), the *C. albicans* $\Delta cdr1/\Delta cdr1$ mutant DSY448 was rendered hypersusceptible to fluconazole, ketoconazole and itraconazole, as well as to other

medically relevant antifungal agents like terbinafine and amorolfine (Vanden Bossche *et al.*, 1994b), and to metabolic inhibitors such as cycloheximide (a protein biosynthesis inhibitor; Mutoh *et al.*, 1995), brefeldin A (an inhibitor of organelle assembly; Graham *et al.*, 1993) and cerulenin (an inhibitor of fatty acid biosynthesis; Morisaki *et al.*, 1993). The $\Delta camdr1/\Delta camdr1$ mutant DSY465 showed hypersusceptibility to 4-nitroquinoline-N-oxide (a mutagen; Decottignies *et al.*, 1995), but not to azole antifungal agents (Goldway *et al.*, 1995; Sanglard *et al.*, 1996). However, when the $\Delta cdr2/\Delta cdr2$ (DSY653) deletion mutant was exposed to the same compounds, the growth of this mutant was not affected (Sanglard *et al.*, 1997).

Some compounds failed to inhibit the growth of the $\Delta flu1/\Delta flu1$ mutants constructed in this study. These substances were: cycloheximide, brefeldin A, cerulenin, benomyl (an antimetabolic drug; Fling *et al.*, 1991), methotrexate (a dihydrofolate reductase inhibitor; Fling *et al.*, 1991), amorolfine, terbinafine and crystal violet (data not shown).

Accumulation of [3 H]fluconazole in *C. albicans*

Since the expression of *FLU1* in *S. cerevisiae* mediates resistance to fluconazole, therefore assigning fluconazole as a substrate for Flu1p, we addressed the accumulation of this azole in *C. albicans* $\Delta flu1/\Delta flu1$ mutants. A similar experiment with the mutant DSY448 revealed a decrease in fluconazole accumulation compared to the wild-type (Sanglard *et al.*, 1996). However, a decrease in fluconazole accumulation could not be measured between $\Delta flu1/\Delta flu1$ mutants and the wild-type, even in the genetic background of multidrug transporter mutants (data not shown). This experiment suggests either a low efflux capacity of Flu1p for fluconazole or low expression levels of this transporter in *C. albicans*, thus making the measurement of accumulation differences between mutants and wild-type cells difficult.

DISCUSSION

We report here the cloning of *FLU1* by functional complementation of fluconazole hypersusceptibility of an *S. cerevisiae* $\Delta pdr5$ mutant. At the outset, the purpose of this procedure was to isolate functional genes to

Fig. 7. Susceptibility of $\Delta flu1/\Delta flu1$ homozygous mutants to antifungal agents and metabolic inhibitors. Each strain was grown to exponential phase to a density of 2×10^7 cells ml^{-1} and $4 \mu\text{l}$ of this suspension was spotted in serial dilution onto YEPD medium agar plates containing different drugs as indicated (concentrations in parentheses are given in $\mu\text{g ml}^{-1}$). Plates were incubated for 48 h at 30°C .

probe their possible involvement in the azole resistance of clinical isolates. This method of complementation permitted the isolation of at least four genes involved in the transport of fluconazole: *CDR1* (Sanglard *et al.*, 1995), *CDR2* (Sanglard *et al.*, 1996), *CaMDR1* (Sanglard *et al.*, 1995) and *FLU1*. Two other genes not related to the family of multidrug efflux transporters were recovered by this strategy, namely *CAP1* and *ERG11* (Sanglard *et al.*, 1997). We cannot exclude that the present screening was not saturated and thus other azole

resistance genes might be cloned in the future using other more drug-sensitive *S. cerevisiae* strains in which additional multidrug efflux transporter genes are deleted. On the other hand, using the data available from the on-going *C. albicans* genome sequencing project, other drug resistance genes belonging to the family of multidrug transporters may be revealed and may participate in the development of azole resistance in the clinical isolates. The rationale of functional complementation has been utilized recently (Launhardt *et al.*, 1998)

for isolating drug resistance genes from *S. cerevisiae* with ketoconazole as a selection pressure. Among the genes recovered were *ERG11*, *COX3* (encoding subunit 1 of cytochrome *c* oxidase), *RPL27* (encoding ribosomal protein L27) and five other genes of unknown function. Thus, these additional genes, although isolated in *S. cerevisiae*, might reveal new pathways by which azole resistance could develop in *C. albicans* clinical isolates. A similar cloning approach would be practical in identifying drug resistance genes from other fungal pathogens of increasing importance, such as *Candida glabrata* or *Candida tropicalis*. We recently isolated three azole resistance genes from *C. glabrata*, among which one, an ABC transporter gene similar to *CDR1*, was up-regulated in azole-resistant isolates (Sanglard *et al.*, 1999).

When expressed in *S. cerevisiae*, *FLU1* mediated specific resistance to fluconazole among the different azole antifungal agents tested. The absence of cross-resistance to all three azole derivatives in *S. cerevisiae* already suggested that the protein encoded by *FLU1* could be more related to the MF *CaMdr1p* than to the ABC transporters *Cdr1p* and *Cdr2p*, since the expression of *CaMDR1* resulted in specific resistance to fluconazole (Sanglard *et al.*, 1995, 1997). In fact, the sequencing of *FLU1* revealed that this gene encoded a putative MF transporter. The structural organization of *Flu1p* was characteristic of MF efflux transporters. MFs have been grouped into three clusters (I, II and III) based on hydropathy analyses. As deduced from this analysis *FLU1* belongs to cluster I, since this transporter has 12 putative transmembrane domains (Goffeau *et al.*, 1997). Further studies should confirm the localization of this protein to the cytoplasmic membrane. *Flu1p* has the highest similarity with YLL028wp from *S. cerevisiae*. This latter transporter might be therefore functional in the efflux of fluconazole or cycloheximide. We attempted to demonstrate this function for YLL028wp; however, although the gene encoding the protein could be expressed under the control of the *ADHI* promoter, no drug resistance phenotype was obtained (data not shown).

In our study, when *FLU1* was up-regulated in the *S. cerevisiae* $\Delta pdr5$ mutant, it increased the resistance of this yeast only to fluconazole and cycloheximide (see Fig. 1a). On the other hand, the disruption of *FLU1* in *C. albicans* did not lead to an increased susceptibility to cycloheximide, but to a small increase in susceptibility to fluconazole. Disruption of *FLU1* did not affect drug susceptibility to a great extent, except in the case of mycophenolic acid. A surprising effect resulting from the disruption of *FLU1* was that a slight increase in susceptibility to all azoles was observed compared to the wild-type, whereas expression of *FLU1* in *S. cerevisiae* could only confer resistance to fluconazole. The most likely explanation of these results could be that the absence of *Flu1p* in *C. albicans* affects either the expression or the activity of other transporters accepting other azoles (itraconazole or ketoconazole) as substrates. Taken together, the results of drug susceptibility

assays reveal differences in two distinct approaches used to assign substrates to specific multidrug transporters. While the activity of transporters in *C. albicans* may be masked by the presence of other transporters responsible for intrinsic resistance of this yeast to various drugs and metabolic inhibitors, these activities may be better demonstrated using *S. cerevisiae* in which the *PDR5* gene was deleted. Alternatively, the differences observed with the two experimental systems may be due to differences in subcellular localization of *Flu1p* between both yeast species or differences in the expression of *FLU1* in both yeast species. We observed that in *C. albicans*, *FLU1* mRNA signals were comparatively very low as opposed to *CDR1* or *TEF3*. In *S. cerevisiae*, *FLU1* was expressed on the support of a YEp24-derived multicopy plasmid. Therefore, expression levels of *FLU1* may have been higher in *S. cerevisiae* and thus could influence the results of the susceptibility assays.

Some ORFs of cluster I MF transporters from different yeast species have known functions as multidrug resistance pumps. *Car1p* of *Schizosaccharomyces pombe* transports cycloheximide and amiloride (Jia *et al.*, 1993), *Cyhrp* from *Candida maltosa* is specific for cycloheximide and hydrophobic drugs (Ben-Yaacov *et al.*, 1994), and *CaMDR1* expression renders *S. cerevisiae* resistant to methotrexate, benomyl, cycloheximide, 4-nitroquinoline-N-oxide, sulfomethuron methyl, terbinafine, amorolfine, fluphenazine, 1,10-phenanthroline, cerulenin and benzotriazoles (Ben-Yaacov *et al.*, 1994; Fling *et al.*, 1991; Sanglard *et al.*, 1997). In *S. cerevisiae*, several MF transporters have been characterized, being functional as multidrug resistant pumps: *Atr1p* transports aminotriazoles and 4-nitroquinoline-N-oxide (Gompel-Klein & Brendel, 1990; Kanazawa *et al.*, 1988), *Sge1p* is specific for crystal violet and ethidium bromide (Amakasu *et al.*, 1993; Ehrenhofer-Murray *et al.*, 1994) and *Flr1p*, which is the functional homologue of *CaMdr1p* in *S. cerevisiae*, can take at least fluconazole and cycloheximide (Alarco *et al.*, 1997) or cerulenin (Oskouian & Saba, 1999) as substrate. The expression of the *S. cerevisiae* MF gene *YOR273c* conferred resistance to the antimalarial drug quinidine (Delling *et al.*, 1998).

Even though *FLU1* encodes a transporter for fluconazole, the expression of this gene in *C. albicans* clinical isolates could not be related to their degree of azole resistance. We observed some variations in the expression of *FLU1* among the clinical isolates investigated in this study, the basis of which remains unknown. This is in contrast to *CaMDR1*, which is up-regulated from almost non-detectable levels to high levels in some azole-resistant isolates. Some indirect evidence could establish a link between the expression of *CaMDR1* and *CAP1*, a recently cloned transcription factor with a basic leucine zipper motif similar to the *S. cerevisiae* *YAP1* gene. *CAP1* was shown to activate the transcription of the MF transporter *FLR1* (Alarco *et al.*, 1997), which is functionally similar to *CaMDR1* in *C. albicans*. Interestingly, the *CaMDR1* promoter contains *YAP1*-like DNA recognition elements: a sequence, 5'-

TGACTCA-3', at position -737 to -731 upstream of the ATG codon, which is the optimal YAP1 binding site (Ellenberger *et al.*, 1992; Kim & Struhl, 1995; Oliphant *et al.*, 1989) and three other sequences that differ at position ± 2 from TGACTCA. Multidrug resistance in *C. albicans* resulting from up-regulation of *CaMDR1* could therefore also be regulated by *CAP1*. *FLU1* does not contain any conventional YAP1-like recognition element in its available promoter sequence and therefore its expression is not likely to be dependent on *CAP1*. These observations are in agreement with the differences in expression between *CaMDR1* and *FLU1* in clinical isolates.

In conclusion, we have shown here that a gene cloned in *S. cerevisiae* by functional complementation for a drug resistance phenotype was not coupled to azole resistance in *C. albicans*. Development of azole resistance can be explained in most azole-resistant strains by known alterations. However, among the growing number of characterized azole-resistant isolates, some of them still acquire resistance by unknown mechanisms. By using a prospective functional screening of *C. albicans* drug resistance genes in suitable yeast genetic backgrounds, these additional remaining azole resistance mechanisms may be revealed in the future.

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