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

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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, fluconazole, 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 14α-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...
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 14α-demethylase), FLU1 and FLU2, the latter now referred to as CAP1 and encoding a transcription factor (Alarcó 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 (\(Δura3::imm434/Δura3:: imm434\)) and the parent CAF2-1 (\(Δura3::imm434/URA3\)) are derived from the wild-type strain SC5314 and were obtained from B. Fonzi (Fonzi & Irwin, 1993). The S. cerevisiae strain YKKB-13 (\(MATα,ura3-52\,lys2-801\,ade2-101\,trp1\,sph1\,bgl2\,trp1\,I\,\(Δ\)SFF1::\(\alpha\)\) was described by Bissinger & Kuchler (1994). The genotypes of other yeast strains 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 µg 5-fluororoorotic acid (5-FOA) ml\(^{-1}\) were made for the regeneration of the ura3 genetic marker in YNB selective medium supplemented with 50 µg uridine ml\(^{-1}\).

**Accumulation of \([^{3}H]fluconazole in C. albicans isolates.** Fluconazole accumulation experiments in C. albicans were performed with \([^{3}H]\)-labelled fluconazole (Amersham) as described previously (Sanglard et al., 1995), except that a single 20 min incubation was used. Each \([^{3}H]\)-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 pD1S1. A blunt-ended 3.7 kb Sall–BglII fragment from pMB-7 (Fonzi & Irwin, 1993) containing the \(his-G\)–URA3–hisG disruption cassette was inserted in the single SmaI site of pD1S1 to create pDIS2. The linear 6.4 kb SphI–BglII fragment from pD1S2 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-hybriddized at 42 °C, with a buffer consisting of 50% formamide, 1% SDS, 4× SSC (1× SSC is 0.15 M sodium chloride, 0.015 M sodium citrate), 10% dextran sulfate and 100 µg salmon sperm DNA ml\(^{-1}\). DNA probes were labelled with \([\alpha]^{32}P\)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 EcoR1–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 digested 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 µg 100T Zymolyase ml\(^{-1}\) (Seikagaku) and 0.1% β-mercapto-ethanol (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 lysis solution (0.1 M EDTA, 0.8% SDS, 50 µg 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 µg RNase A ml\(^{-1}\) (Roche) and incubated at 37 °C for 15 min. DNA from each culture was then precipitated with 2-propanol, transferred to 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× SSC, 1%
Table 1. Genotypes of yeast strains used in this study

<table>
<thead>
<tr>
<th>Strain</th>
<th>Parental strain</th>
<th>Genotype</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>CAF4-2</td>
<td>CAF2-1</td>
<td>Δura3::immr434/Δura3::immr434</td>
<td>Fonzi &amp; Irwin (1993)</td>
</tr>
<tr>
<td>DSY448</td>
<td>DSY447</td>
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<td>Sanglard et al. (1996)</td>
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<tr>
<td>DSY465</td>
<td>DSY464</td>
<td>Δcamdr1::hisG-URA3-hisG/Δcamdr1::hisG</td>
<td>Sanglard et al. (1996)</td>
</tr>
<tr>
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<td>DSY467</td>
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<td>Sanglard et al. (1996)</td>
</tr>
<tr>
<td>DSY653</td>
<td>DSY465</td>
<td>Δacr2::hisG-URA3-hisG/Δacr2::hisG</td>
<td>Sanglard et al. (1997)</td>
</tr>
<tr>
<td>DSY654</td>
<td>DSY653</td>
<td>Δacr2::hisG/Δacr2::hisG</td>
<td>Sanglard et al. (1997)</td>
</tr>
<tr>
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<td>DCA-2</td>
<td>Δflur1::hisG-URA3-hisG/FLU1</td>
<td>This study</td>
</tr>
<tr>
<td>DCY2</td>
<td>DBC-1</td>
<td>Δflur1::hisG/FLU1</td>
<td>This study</td>
</tr>
<tr>
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<td>This study</td>
</tr>
<tr>
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<td>DCSY465</td>
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<td>This study</td>
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<td>Δcamdr1::hisG/Δcamdr1::hisG</td>
<td>This study</td>
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<td>DCSY465</td>
<td>Δcamdr1::hisG/Δcamdr1::hisG</td>
<td>This study</td>
</tr>
</tbody>
</table>

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

<table>
<thead>
<tr>
<th>Patient no.</th>
<th>C. albicans isolate</th>
<th>MIC (µg ml⁻¹)</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Fluconazole</td>
<td>Ketoconazole</td>
</tr>
<tr>
<td>I</td>
<td>C27</td>
<td>1</td>
<td>0.015</td>
</tr>
<tr>
<td></td>
<td>C40</td>
<td>128</td>
<td>2</td>
</tr>
<tr>
<td>II</td>
<td>C33</td>
<td>0.25</td>
<td>0.015</td>
</tr>
<tr>
<td></td>
<td>C26</td>
<td>&gt; 128</td>
<td>4</td>
</tr>
<tr>
<td>III</td>
<td>C43</td>
<td>0.25</td>
<td>0.015</td>
</tr>
<tr>
<td></td>
<td>C56</td>
<td>128</td>
<td>4</td>
</tr>
<tr>
<td>IV</td>
<td>26</td>
<td>0.25</td>
<td>0.031</td>
</tr>
<tr>
<td></td>
<td>91</td>
<td>16</td>
<td>0.031</td>
</tr>
<tr>
<td>V</td>
<td>70</td>
<td>1</td>
<td>0.002</td>
</tr>
<tr>
<td></td>
<td>74</td>
<td>16</td>
<td>0.125</td>
</tr>
<tr>
<td>VI</td>
<td>741</td>
<td>0.25</td>
<td>0.015</td>
</tr>
<tr>
<td></td>
<td>742</td>
<td>16</td>
<td>0.062</td>
</tr>
</tbody>
</table>

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

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 was derived 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
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...
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 Δflu1/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 Δflu1::hisG-URA3-hisG allele and 1.84 kb for the disrupted allele after 5-FOA treatment (Δflu1::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/Δflu1::hisG). The 4.7 kb band, which was expected for the Δflu1::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 Δflu1 mutants (DCY2, DCY6, DCY10, DCY13, DCY16 and DCY19) and homozygous Δflu1 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 Δflu1::hisG allele after 5-FOA regeneration and 8.2 kb for the Δflu1::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 Δflu1::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
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 ∆flu1/∆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 (∆cdr1/∆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 ∆flu1/∆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 ∆cdr1/∆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 cerulениn (an inhibitor of fatty acid biosynthesis; Morisaki et al., 1993). The ∆camdr1/∆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 ∆cdr2/∆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 ∆flu1/∆flu1 mutants constructed in this study. These substances were: cycloheximide, brefeldin A, cerulениn, benomyl (an antimitotic drug; Fling et al., 1991), methotrexate (a dihydrofolate reductase inhibitor; Fling et al., 1991), amorolfine, terbinafine and crystal violet (data not shown).

Accumulation of [3H]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 ∆flu1/∆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 ∆flu1/∆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 ∆pdr5 mutant. At the outset, the purpose of this procedure was to isolate functional genes to
probe their possible involvement in theazole resistance of clinical isolates. This method of complementation permitted the isolation of at least four genes involved in the transport of fluconazole: CDRI (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 ofazole 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 I 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 differentazole antifungal agents tested. The absence of cross-resistance to all threeazole 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 *YOR273C* 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), Cdr1p from *Candida maltosa* is specific for cycloheximide and hydroporphic drugs (Ben-Yaacov et al., 1994), and *CaMDR1* expression renders *S. cerevisiae* resistant to methotrexate, benomyl, cycloheximide, 4-nitroquinoline-N-oxide, sulframethuron methyl, terbinafine, amorolfine, flufenazime, 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: At1p transports aminomirotioazoles 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 CaMDRI 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 CaMDRI 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.

ACKNOWLEDGEMENTS

This work was supported by a grant No 3100-04716 from the Swiss National Foundation to D.S. Our thanks to F. Ischer for excellent technical assistance.

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Received 31 March 2000; revised 30 June 2000; accepted 20 July 2000.