Cloning of *Candida albicans* genes conferring resistance to azole antifungal agents: characterization of *CDR2*, a new multidrug ABC transporter gene

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Resistance to azole antifungal agents in *Candida albicans* can be mediated by multidrug efflux transporters. In a previous study, we identified at least two such transporters, Cdr1p and Benp, which belong to the class of ATP-binding cassette (ABC) transporters and of major facilitators, respectively. To isolate additional factors potentially responsible for resistance to azole antifungal agents in *C. albicans*, the hypersusceptibility of a *Saccharomyces cerevisiae* multidrug transporter mutant, Δpdr5, to these agents was complemented with a *C. albicans* genomic library. Several new genes were isolated, one of which was a new ABC transporter gene called *CDR2* (*Candida drug Eesistance*). The protein Cdr2p encoded by this gene exhibited 84% identity with Cdr1p and could confer resistance to azole antifungal agents, to other antifungals (terbinafine, amorolfine) and to a variety of metabolic inhibitors. The disruption of *CDR2* in the *C. albicans* strain CAF 4-2 did not render cells more susceptible to these substances. When the disruption of *CDR2* was performed in the background of a mutant in which *CDR7* was deleted, the resulting double Δcdr1 Δcdr2 mutant was more susceptible to these agents than the single Δcdr1 mutant. The absence of hypersusceptibility of the single Δcdr2 mutant could be explained by the absence of *CDR2* mRNA in azole-susceptible *C. albicans* strains. *CDR2* was overexpressed, however, in clinical *C. albicans* isolates resistant to azole antifungal agents as described previously for *CDR1*, but to levels exceeding or equal to those reached by *CDR1*. Interestingly, *CDR2* expression was restored in Δcdr1 mutants reverting spontaneously to wild-type levels of susceptibility to azole antifungal agents. These data demonstrate that *CDR2* plays an important role in mediating the resistance of *C. albicans* to azole antifungal agents.

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

INTRODUCTION

*Candida albicans* is an important opportunistic pathogen which can cause disseminated and mucosal infections in humans (Odds *et al.*, 1990). Patients with compromised immune systems, for example, patients receiving organ transplants and cancer chemotherapy or people infected by HIV, who see their immune system gradually affected by their illness, are particularly prone to such infections. *C. albicans* is the major agent of oropharyngeal candidiasis in AIDS patients. Oropharyngeal candidiasis is one of the most frequent fungal infections of recent years, since it occurs in up to 90% of AIDS patients during their life span (Vanden Bossche *et al.*, 1994a). Among antifungal agents available to treat infections caused by *C. albicans*, fluconazole is by far the most commonly used compound (Powderly, 1994). The cellular target of fluconazole and other azole derivatives in yeast is a cytochrome P450 (CYP51A1),

**Abbreviations**: ABC transporter, ATP-binding cassette transporter; 5-FOA, 5-fluoroorotic acid.

The GenBank accession number for the sequence reported in this paper is U63812.
which is a haemoprotein involved in the 14α-demethylation of lanosterol, an important step in the biosynthesis of ergosterol (Vanden Bossche et al., 1994b). As a consequence of the increasing number of infections caused by C. albicans, the use of this antifungal agent has been more widespread. Repeated treatments with fluconazole have led to the appearance of yeast isolates resistant to this agent in vitro (Johnson et al., 1995b; Odds, 1996; Rex et al., 1995; Vuffray et al., 1994). In a recent study investigating the mechanisms of resistance to fluconazole in C. albicans isolates from AIDS patients with oropharyngeal candidiasis, we could demonstrate that the majority of resistant C. albicans isolates failed to accumulate the intracellular levels of fluconazole reached in susceptible isolates. This phenomenon could be correlated with an enhanced fluconazole efflux. Two multidrug efflux transporters, the ATP-binding cassette (ABC) transporter Cdr1p and the major facilitator Bep1, were identified as possible mediators in this process (Sanglard et al., 1995). The genes for these transporters, CDR1 and BEN1, have been isolated by Prasad et al. (1995) and Fling et al. (1991) and also in our laboratory by complementation of fluconazole hypersusceptibility of a Saccharomyces cerevisiae mutant lacking the ABC transporter Pdr3p/Sts1p (Sanglard et al., 1995).

We expected that other factors involving efflux drug transport or other mechanisms could be responsible for the resistance of C. albicans to azole antifungal agents. A number of other mechanisms involving alterations of the cellular target, CYP51A1, or alterations in the ergosterol biosynthetic pathway have been proposed (Marichal & Vanden Bossche, 1995). Rather than comparing the physiology and biochemistry of azole-susceptible and azole-resistant C. albicans isolates, a more global strategy enabling the cloning of any C. albicans DNA sequences rendering the S. cerevisiae multidrug transporter mutant Δpdr5 resistant to these agents was developed. The possible involvement of cloned genes in the resistance toazole antifungal agents could then be addressed in a panel of clinical isolates. Here, the results obtained using such a strategy and an extended characterization of one of the cloned genes, which was similar to CDR1 and therefore named CDR2, are reported.

METHODS

Strains. C. albicans CAF4-2 (Δura3::imm434/Δura1::imm434) and the parent CAF2-1 (Δura3::imm434/URA3) were from B. Fonzi (Fonzi & Irwin, 1993). The S. cerevisiae strain YKKB13 (MATα ura3-52 lys2-801amber ade2-101* trpl-A63 his3ΔA200 len2-D1 Δsts1::TRP1) has been described by Bissinger & Kuchler (1994). The genotypes of other yeast strains are listed in Table 1. E. coli DH5z (Hanahan, 1985) was used as a host for plasmid construction and propagation.

Media. C. albicans strains were grown on YEPD complex medium with 2% glucose, 1% Bacto peptone (Difco) and 0.5% yeast extract (Difco). YEPD agar plates contained 2% agar (Difco) as a supplement. Yeast Nitrogen Base (YNB; Difco) with 2% glucose and 2% agar (Difco) was used as a selective medium after transformation of C. albicans. Agar plates containing 50 μg 5-fluoroorotic acid (5-FOA) ml⁻¹ were made for the regeneration of the ura3 genetic marker in YNB selective medium with 50 μg uridine ml⁻¹.

Drug susceptibility testing. Susceptibility of C. albicans strains to different compounds was tested qualitatively by spotting serial dilutions of yeast cultures in complex YEPD medium agar plates, which provides an easy visualization of growth differences between different yeast strains. The following drugs were solubilized in DMSO: ketoconazole and itraconazole (Janssen Pharmaceuticals), terbinafine (Sandoz Pharma), and nocardazole, brefeldin A, cerulenin and 4-nitroquinoline N-oxide (Sigma). Fluconazole (Pfizer), amolofine chloride (Hoffmann-La Roche) and crystal violet (Sigma) were dissolved in water. Each plate contained 15 ml agar. The drugs were diluted in the corresponding solvents to achieve the concentrations used in YEPD plates. Preliminary tests were performed to optimize drug concentrations in YEPD plates so that growth differences between the different C. albicans strains used in this study could be observed. To perform the susceptibility tests, yeasts were grown overnight at 30°C with constant shaking in YEPD liquid medium. The cultures were diluted to 2 x 10⁷ cells ml⁻¹ in 0.9% NaCl. Five microlitres of this suspension and of serial dilutions of each yeast culture were spotted on each type of plate and incubated for a given time at 30°C.

Accumulation of fluconazole in yeast. Accumulation experiments of [³H]fluconazole (Amersham) in C. albicans CAF2-1 and in multidrug transporter mutants were performed as described previously (Sanglard et al., 1995), except that 20 min incubation was chosen as the single sampling time. Each accumulation experiment was repeated twice.

Construction of plasmids. For the disruption of CDR2 in C. albicans, a 24 kb Spel-KpnI fragment from pDS346 was subcloned in pBluescript KS(+) to yield pDS346. An internal 0.9 kb HindIII-PstI fragment from pDS346 was replaced by the hisG-URA3-hisG disruption cassette from pMB7 (Fonzi & Irwin, 1993) to create pDS368. The linear 5.2 kb Spel-KpnI fragment from pDS368 was used for disruption experiments.

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

Yeast transformation. For gene disruptions, C. albicans CAF4-2 was transformed with linear fragments by an LiAc procedure developed in our laboratory. Yeast were grown to mid-exponential phase to a density of 2 x 10⁹ cells ml⁻¹ in 100 ml YEPD complex medium with constant shaking at 250 r.p.m. Fifty millilitres of this culture was removed to a 50 ml Falcon tube and centrifuged at 5000 r.p.m. at 4°C for 5 min. The pellet was washed twice with TE buffer (10 mM Tris/HCl, pH 7.5; 1 mM EDTA). Another washing was performed with 10 ml Li/TE buffer (0.1 M LiAc in TE, pH 7.5). The final cell pellet was resuspended in 200 μl Li/TE buffer and placed on ice. Yeast suspension (50 μl) was aliquoted into Eppendorf tubes. The following reagents were added sequentially to each tube: 300 μl of freshly prepared PEG buffer (40% PEG 4000 in Li/TE buffer), 5 μl 10 mg sheared herring sperm DNA (Clontech) ml⁻¹, then linear DNA fragments (1–2 μg) in a maximum volume of 5 μl. The tubes were mixed gently and incubated for 30 min at 30°C. After heat shock at 42°C for 20 min, the tubes were centrifuged briefly three times in a microcentrifuge at room temperature.
The supernatant was removed and the cell pellet was resuspended in 200 µl TE. Aliquots of this suspension were plated in YNB selective medium and incubated for 3-4 d at 30 °C.

**Plasmid rescue from *S. cerevisiae***. Episomal plasmids from the parent vector YEp24 were rescued from *S. cerevisiae* transformants by electroporation. Yeast were grown in selective YNB medium to late-exponential phase and total DNA was extracted as outlined below. DNA suspension (1 µl) was electroporated into *E. coli* DH5α and ampicillin-resistant clones from each transformant were analysed by restriction enzyme analysis.

**Chromosome and RNA electrophoresis, Northern blot hybridizations and quantification.** Chromosomes of *C. albicans* were separated for gene mapping by the clamped homogeneous electric field technique (CHEF-DRII; Bio-Rad) as described by Monod et al. (1994).

For Northern blots, total RNA from yeast was extracted and electrophoresed following the method described in Sanglard et al. (1995). Transfer of RNA was performed by capillarity on Genescreen Plus membranes (DuPont NEN). Membranes were pre-hybridized at 42 °C with a buffer consisting of 50% formamide, 1% SDS, 4 x SSC, 10% dextran sulfate and 100 µg salmon sperm DNA ml−1. [32P]DNA-labelled probes were generated by random priming (Feiberg & Vogelstein, 1984) and added to the hybridization solution overnight. Washing steps were at high stringency, identical with those recommended by the supplier (DuPont NEN). In Northern blots, the TEF3 mRNAs were analysed using a 0.7 kb EcoRI–PstI fragment from pDC1 as described by Hube et al. (1994). Stripping of probes in sequential hybridizations was achieved by boiling the membranes for 10 min in 0.1% SDS in TE buffer. Details about other probes are described in the legend of the corresponding figures.

**Quantifications of Northern blots** were performed by exposure of the hybridized membranes in an Instant Imager (Packard Instrument). Signals were integrated by the software supplied by the manufacturer and normalized to the corresponding values of the TEF3 internal standard.

**PCR.** PCR buffers and AmpliTaq polymerase were from Perkin Elmer (Roche Molecular Systems). The buffer composition was 10 mM Tris/HCl (pH 8.3), 50 mM KCl and 1.5 mM MgCl₂ containing 0.2 mM dNTPs and 2.5 U polymerase per reaction. Briefly, the 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 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 Fig. 5 and were designed on the basis of the *C. albicans* CDR2 and *Salmonella typhimurium* hisG nucleotide sequences in the GenBank database.

Yeast DNA templates for PCR were prepared from overnight cultures on complex medium. A 1 ml portion of these cultures was centrifuged in an Eppendorf tube and DNA was extracted by adding 0.3 g glass beads, 200 µl of a breaking buffer (2% Triton X-100, 1% SDS, 10 mM Tris/HCl, pH 8.0, 1 mM EDTA, 100 mM NaCl) and 200 µl phenol/chloroform/isoamyl alcohol (24:1.1, v/v). After 1 min vortexing, the tubes were centrifuged at maximum speed for 10 min in the microcentrifuge and the supernatant was re-extracted with chloroform/isoamyl alcohol (24:1, v/v). Nucleic acids were then precipitated with ethanol and resuspended in 50 µl TE. Of this suspension, 1 µl was used for PCR.

**RESULTS**

Cloning of *C. albicans* genes conferring resistance to azole antifungal agents

We reported recently than an *S. cerevisiae* mutant YKBB-13 lacking the ABC transporter Pdr5p was hypersusceptible toazole antifungal agents (Sanglard et al., 1995). This phenotype seemed to be adequate for complementing hypersusceptibility with any *C. albicans* DNA segment carrying genes of resistance toazole antifungal agents. These genes are thus likely to encode multidrug efflux transporters; however, they may belong to totally unrelated genes. A *C. albicans* genomic library from strain SC5314 was therefore constructed in the episomal vector YEp24 (Sanglard et al., 1995) and the resulting library was transformed in the *S. cerevisiae* strain YKBB-13. A total of 40000 Ura⁺ clones was obtained and spotted in selective YNB plates containing in a first screening 10 µg fluconazole ml⁻¹. Thirty-two azole-resistant clones emerged and their plasmids were

**Table 1. Genotypes of yeast strains used in this study**

<table>
<thead>
<tr>
<th>Strain name</th>
<th>Parent strain</th>
<th>Genotype</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>CAF2-1</td>
<td>SC5314</td>
<td>Δura3::imm434/URA3</td>
<td>Fonzi &amp; Irwin (1993)</td>
</tr>
<tr>
<td>CAF4-2</td>
<td>CAF2-1</td>
<td>Δura3::imm434/Δura3::imm434</td>
<td>Fonzi &amp; Irwin (1993)</td>
</tr>
<tr>
<td>DSY448</td>
<td>DSY447</td>
<td>Δacr1::hisG–URA3–hisG</td>
<td>Sanglard et al. (1996)</td>
</tr>
<tr>
<td>DSY449</td>
<td>DSY448</td>
<td>Δacr1::hisG/Δacr1::hisG</td>
<td>Sanglard et al. (1996)</td>
</tr>
<tr>
<td>DSY649</td>
<td>CAF4-2</td>
<td>Δacr2::hisG–URA3–hisG</td>
<td>This study</td>
</tr>
<tr>
<td>DSY651</td>
<td>DSY649</td>
<td>Δacr2::hisG/CDR2</td>
<td>This study</td>
</tr>
<tr>
<td>DSY653</td>
<td>DSY651</td>
<td>Δacr2::hisG–URA3–hisG</td>
<td>This study</td>
</tr>
<tr>
<td>DSY650</td>
<td>DSY449</td>
<td>Δacr1::hisG/Δacr1::hisG</td>
<td>This study</td>
</tr>
<tr>
<td>DSY652</td>
<td>DSY650</td>
<td>Δacr1::hisG/Δacr1::hisG</td>
<td>This study</td>
</tr>
<tr>
<td>DSY654</td>
<td>DSY652</td>
<td>Δacr1::hisG/Δacr1::hisG</td>
<td>This study</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Δacr2::hisG–URA3–hisG/Δacr2::hisG</td>
<td>This study</td>
</tr>
</tbody>
</table>
Growth was recorded as positive by the presence of confluent colonies or single colonies with a visible diameter after 2 d incubation at 30 °C. Spotting of cells on plates was performed as described in Methods for drug susceptibility assays. Values: 4, growth at dilution 10^-4; 3, growth at 2; growth at 1; growth at 10^-1; 0, growth at 10^-0; −, no growth at 10^-9.

<table>
<thead>
<tr>
<th>Substance*</th>
<th>Plasmid</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>YEp24</td>
</tr>
<tr>
<td>YePD only</td>
<td>4</td>
</tr>
<tr>
<td>Flu (5)</td>
<td>-</td>
</tr>
<tr>
<td>Itra (0.025)</td>
<td>-</td>
</tr>
<tr>
<td>Keto (0.05)</td>
<td>-</td>
</tr>
<tr>
<td>Cyh (0.025)</td>
<td>-</td>
</tr>
<tr>
<td>NQO (50)</td>
<td>-</td>
</tr>
<tr>
<td>Ben (50)</td>
<td>-</td>
</tr>
<tr>
<td>Terb (8)</td>
<td>0</td>
</tr>
<tr>
<td>Amo (0.05)</td>
<td>-</td>
</tr>
<tr>
<td>Sum (50)</td>
<td>0</td>
</tr>
<tr>
<td>Flup (10)</td>
<td>-</td>
</tr>
<tr>
<td>Phe (10)</td>
<td>-</td>
</tr>
<tr>
<td>Rho (5)</td>
<td>-</td>
</tr>
<tr>
<td>Cer (0.5)</td>
<td>0</td>
</tr>
<tr>
<td>Bref (25)</td>
<td>2</td>
</tr>
<tr>
<td>Cry (0.05)</td>
<td>-</td>
</tr>
</tbody>
</table>

* Abbreviations for substances are followed by a number giving the concentration in μg ml⁻¹ used in YEpd medium. Amo, amorolfine; Ben, benomyl; Bref, brefeldin A; Cer, cerulenin; Cry, crystal violet; Cyh, cycloheximide; Itra, itraconazole; Flu, fluconazole; Flup, fluphenazine; Keto, ketoconazole; NQO, 4-nitroquinoline N-oxide; Phe, 1,10-phenanthroline; Rho, rhodamine 6G; Sum, sulfmethuron methyl; Terb, terbinafine.

† pDS270 was isolated by ketoconazole-resistance selection.

rescued in *E. coli* DH5α by electroporation. Plasmids with unique and not overlapping restriction sites were selected and re-transformed in YKKB-13. After this selection, five different plasmids, each potentially carrying a unique resistance gene, were isolated. The same screening procedure was used with 0.2 μg ketoconazole ml⁻¹ in selective YNB medium. This procedure yielded plasmids, some of them with the same profiles as those obtained by fluconazole selection. However, one additional unique plasmid with a resistance gene was isolated, bringing the total number of resistance genes to six. Table 2 summarizes the pattern of resistance produced by each of these plasmids in YKKB-13, not only toazole antifungal agents but also to other antifungals and different metabolic inhibitors. All plasmids conferred resistance to fluconazole but to different degrees; for example, the presence of pDS243, pDS239 and pDS255 in YKKB-13 allowed cells to grow at all dilutions tested in a medium with 5 μg fluconazole ml⁻¹ whereas cells containing pDS246, pDS237 and pDS270 exhibited visible growth only up to dilutions of 10^-2 and 10^-1. While pDS239, pDS255 and pDS257 yielded specific resistance to fluconazole, only pDS243, pDS246 and pDS270 could confer cross-resistance to all threeazole derivatives used in this study, but to different extents. pDS243 and pDS239, whose restriction maps are shown in Fig. 1, have been reported in a previous study and contained the *CDR1* and *BEN1* genes (Sanglard et al., 1993). *CDR1* had previously been cloned by Prasad et al. (1995) by complementation of cycloheximide hypersusceptibility of an *S. cerevisiae* Δ*др5* mutant, while *BEN1* was cloned by Fling et al. (1991) in experiments where resistance to benomyl was conferred to another *S. cerevisiae* strain. The pattern of resistance of yeast transformed by the *CDR1*-containing plasmid pDS243 covers a wide range of substances (Table 2). This feature is typical of multidrug transporters of the ABC family, but among these substances are amorolfine and terbinafine, which are antifungals of medical relevance (Vanden Bossche et al., 1994b), sulfmethuron methyl, rhodamine 6G, cerulenin and brefeldin A. These compounds have not yet been reported as substrates for this transporter. pDS239, which contained *BEN1*, besides conferring resistance to already reported substances such as cycloheximide, benomyl and 4-nitroquinoline N-oxide (Goldway et al., 1995), could also accept as new substrates terbinafine, sulfmethuron methyl, 1,10-phenanthroline, cerulenin and brefeldin A (Table 2). Among these new substrates, terbinafine, sulfmethuron methyl, cerulenin and brefeldin A can be used as substrates by both multidrug transporter classes. Specific substrates for *BEN1* are...
features of typical yeast promoters: an adenosine at
observed in this fragment. The 5'-flanking region
Interestingly, in the 3'-flanking region of the nucleotide
2, namely pDS255, pDS257 and pDS270, contained,
Sequence analysis of CDR2
A 5800 bp NlaIV-EcoRI fragment from pDS246, which
5'-flanking region
pDS246 conferred resistance to all azole derivatives, as
mentioned above, and to a very similar variety of other
metabolic inhibitors as CDR1, but with less potency
than this gene. However, pDS246 seemed to confer a
specific resistance to crystal violet (Table 2). Restriction
mapping of pDS246 was performed (Fig. 1) and sequence
analysis showed that an ORF encoding 1499 aa was
present in the insert of this plasmid (see also Fig. 2). This
ORF had a high identity (84%) to that in CDR1 (Prasad
et al., 1995) and therefore it was named CDR2.
The genes carried by the other plasmids listed in Table
2, namely pDS255, pDS257 and pDS270, contained,
according to the data available from total or partial
nucleotide sequences, unidentified C. albicans genes
when compared with the current GenBank database
(this will be reported elsewhere). In this study, we
focused only on CDR2, since it is closely related to
CDR1 and is possibly involved in resistance to azole
antifungal agents in clinical C. albicans isolates.

Sequence analysis of CDR2
A 5800 bp NlaIV–EcoRI fragment from pDS246, which
was expected from partial nucleotide sequence analysis
to contain the entire CDR2 gene, was subcloned with
available restriction sites and its nucleotide sequence
was determined. An uninterrupted ORF of 4500 bp
starting from the most upstream ATG codon was
observed in this fragment. The 5'-flanking region
starting from this ATG codon displayed conserved
features of typical yeast promoters: an adenine at
position -3 and a TATA box at position -111 (Chen
& Struhl, 1988) could be clearly distinguished.
Interestingly, in the 3'-flanking region of the nucleotide
sequence in Fig. 2, an ORF beginning at +4850 has high
similarity with the ORF e242682 of unknown function
in S. cerevisiae.

CDR2 maps, as in the case of CDR1, on chromosome 3
of the C. albicans genome, as determined by chromo-
some separation by the clamped homogeneous electric
field technique and by mapping on a fosmid database (S.
Scherer, personal communication; data available at
URL http://alces.med.umn.edu/bin/genelist/genes).
The CDR2 ORF encodes a protein of 168 kDa and
displays a structure and domain organization typical of
membrane proteins of the ABC superfamily. It is 84%
identical and 92% similar to Cdr1p; however, alignment
of the two amino acid sequences shows that the N-
and C-terminal portions of both proteins were more
divergent (Fig. 3). As in the case of Cdr1p, Cdr2p is
composed of two homologous halves, each comprising a
C-hydrophilic and an N-hydrophobic domain. The
hydrophathy plot of Cdr2p identified six putative trans-
membrane domains for each of the hydrophobic
domains (see Fig. 2). Each hydrophilic domain included
ATP-binding motifs found in ATP-binding cassette
domains. In the N-terminal ABC domain, the Walker A
(GRPGAGCST) and B (IQCVD) motifs and the ABC
signature (VSGGERKRVSIA) are identical in both
proteins. In the C-terminal ABC domain, both the
Walker A (GASGAGKT) and Walker B (LLFLD)
regions are present in both proteins, but no typical ABC
signature is present.
The high similarity between the amino acid sequences
of Cdr1p and Cdr2p does not, however, extend to the
regions flanking the ORF of both genes. The 5'-flanking
and promoter regions of both genes are especially
divergent and thus suggest that these genes may differ in
their regulation.

Expression of CDR2 in clinical C. albicans isolates
To address the possible expression of CDR2 in C.
albicans, two pairs of C. albicans clinical isolates were
selected, each of which contained closely related isolates
susceptible and resistant toazole antifungal agents.
These strains were isolated from two different AIDS
patients with oropharyngeal candidiasis and were
analysed in a previous study in which the resistant
isolate of each pair was reported to overexpress CDR1
(Sanglard et al., 1995). Total RNA from the isolates was
extracted and subjected to Northern blot analysis with
probes specific for CDR1 and CDR2. These probes were
of identical length and spanned the same 5'-flanking
regions of both genes, where the degree of homology between the two genes is very low. Thus, no cross-hybridization between the two probes could be expected. As presented in Fig. 4, CDR1 was expressed constitutively but at low levels in strains still susceptible to azole antifungal agents and, as expected, increased expression was observed when the isolates of each pair became resistant to the same agents. Quantifications with the Instant Imager (see Methods) revealed that the expression of CDR1 was increased in C34 over C26 and in C56 over C43 by 2.7-fold and 4.5-fold, respectively. Conversely, CDR2 was not expressed in the same susceptible isolates, and most surprisingly, this gene was over-expressed when the isolates became resistant to azole antifungal agents. The relative CDR2 mRNA levels exceeded those of CDR1 by a factor of 6 in isolate C26 and only slightly those of CDR1, by a factor of 1.3, in isolate C56.
proteins Cdr1p and Cdr2p. Identical amino acid residues are

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**Fig. 3.** Alignment of the *C. albicans* multidrug resistance proteins Cdr1p and Cdr2p. Identical amino acid residues are marked by a bar. Colons and periods indicate high and low conservative replacements between the two proteins. Walker A and B domains and the ABC signature are underlined.

**Fig. 4.** Northern blot analysis of *C. albicans* clinical isolates susceptible and resistant to azole antifungal agents. Isolates C34 and C26, and C43 and C56 are azole-susceptible and azole-resistant pairs from two distinct patients. These strains have been characterized and have been tested for their susceptibility to fluconazole, itraconazole and ketoconazole as described previously (Sanglard et al., 1995). The Northern blot was probed sequentially with CDR1 and CDR2 probes and finally a TEF3 probe as an internal standard for normalization of the loaded quantities. Specific CDR1 and CDR2 probes were generated by PCR with primers spanning the nucleotide regions -109 to +280 and -112 to +274 with respect to the first ATG initiation codon of the published sequences. The identity between the two probes at the level of the nucleotide sequence is below 50%. The figure shows the signal intensities obtained with the CDR1 and CDR2 probes after 9 and 18 h exposure of membranes on an RX Fuji X-ray film at -70°C. Signal intensities with the TEF3 probe were obtained after 3 h exposure on the same X-ray film.

**Disruption of the CDR2 gene**

To investigate the effect of Cdr2p in *C. albicans*, the gene encoding this protein was deleted by targeted gene disruption by the method described by Fonzi & Irwin (1993). CAF4-2, and also a strain named DSY449 (Table 1) in which both alleles of CDR1 were deleted (Sanglard et al., 1996), were utilized as recipients for transformation with a linear fragment in which a substantial region of CDR2 was deleted and replaced by the *hisG–UR3–hisG* URA3–blaster disruption cassette (see Fig. 5). An LiAc transformation protocol was utilized here and yielded approximately 100 Ura+ transformants (μg linear fragment)-1. A PCR method was applied in this study to screen Ura+ transformants for the correct localization of the disruption of the first CDR2 allele. This approach consisted of using a pair of primers where one was specific for the *hisG* gene and the other allele.

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Fig. 5. Restriction maps of the CDR2 alleles in disruption experiments. Parts I and II are schematic representations of the disruption of the CDR2 gene before and after regeneration of the ura3 genetic marker by selection with 5-FOA. The maps of the wild-type alleles are shown with the corresponding disruption of the fragment containing the gene deletion construct as indicated. The linear fragment containing the gene deletion construct as represented in panel I and which was used for disruption experiments was the 5.2 kb SpeI-KpnI fragment from pDS368.

(DSY649 and DSY650) is shown in Fig. 6. As expected, PCR performed with primers A (specific for the CDR2 gene) and B (specific for the hisG gene) with genomic DNA of these strains yielded a major PCR product of 1.6 kb. One positive transformant of each first allele disruption was further treated with 5-FOA to regenerate the ura3 genetic marker. The regeneration of the ura3 marker by recombination between hisG repeats rather than by mitotic recombination between the wild-type allele and the first disrupted alleles was verified on 5-FOA-resistant strains by PCR analysis. In each case, four 5-FOA-resistant clones were analysed and all had the desired genotype. Only PCR analysis of one representative of each marker regeneration (DSY651 and DSY652) is shown in Fig. 6. PCR products from DSY651 and DSY652 using primers A and C (both specific for CDR2) were obtained with sizes of 2.1 and 2.4 kb, corresponding to the sizes expected from the wild-type allele and the Δcdr2::hisG disrupted allele, respectively.

The linear fragment containing the CDR2 deletion construct was then re-used for the disruption of the second CDR2 allele in both cases. PCR analysis was used again to select Ura" transformants where both alleles were disrupted. On this selection basis, the Ura" Δcdr2 and Δcdr1 Δcdr2 homozygous deletion mutants DSY653 and DSY654 could be successfully isolated. Only 2 out of 10 Ura" transformants in the case of the CDR2 disruption in the CAF4-2 genetic background and only one 1 out of 33 Ura" transformants in the case of the CDR2 disruption in the Δcdr1 deletion mutant background had this correct genotype. The other transformants in both cases were still heterozygous for the gene deletions, since homologous recombination of the disruption cassette with the first disrupted Δcdr2::hisG alleles rather than with the wild-type CDR2 alleles had occurred in both cases. Verification of the gene deletions in DSY653 and DSY654 was performed with Southern blot analysis using hisG as a probe. No signals other than those expected by the gene replacement of the wild-type alleles by the disrupted alleles were observed in this analysis, thus confirming the results obtained by PCR analysis with respect to the identification of the constructed mutants (data not shown). The Δcdr2 homozygote deletion mutants created were then used in drug susceptibility assays.

Drug susceptibility assays with Δcdr2 and Δcdr1 Δcdr2 mutants

Recently, the usefulness of multidrug transporter mutants for addressing the function of multidrug transporters and for assigning unrelated compounds as possible substrates of these proteins in C. albicans was reported (Sanglard et al., 1996). Since many of these compounds inhibit the growth of C. albicans, it is therefore possible to assign them to putative substrates of multidrug transporters by observing growth variations between different yeast types on agar plates containing the different inhibitors. A reduced growth of a given mutant in the presence of a defined inhibitor can be the consequence of an increased accumulation of the substrate due to the absence of its specific efflux transporter. The previously constructed Δcdr1 mutant DSY448 was shown in fact to be hypersusceptible, not only to azole antifungal agents, but also to other metabolic inhibitors (Sanglard et al., 1996). Fig. 7 presents these features and one can observe that every listed compound affected, but to different extents, the growth of DSY448 compared to the wild-type CAF2-1. When the Δcdr2 deletion mutant DSY653 was exposed to the same array of compounds listed in Fig. 7, however, the growth of this mutant was surprisingly not affected. Only when the CDR2 gene deletion is constructed in a Δcdr1 background can a more severe growth inhibition be observed compared to the single Δcdr1 deletion mutant for almost every compound tested. Exceptions were found in plates containing nocodazole and crystal violet, where no difference in growth was found between the single Δcdr1 and the double Δcdr1 Δcdr2 mutants. Intracellular concentrations of [3H]fluconazole were measured in these mutants to illustrate these observations, as indicated in Methods. Whereas the Δcdr2 deletion mutant DSY653 accumulated approximately the same levels of fluconazole as the wild-type (670 ± 25 and 718 ± 21 c.p.m. per 10⁶ cells, respectively), accumulation in the Δcdr1 and the double Δcdr1 Δcdr2 mutants was increased by a factor of 2.3 and 3.1. These data are consistent with an increased susceptibility to fluconazole...
of the Δcdr1 Δcdr2 mutant DSY654 compared to the Δcdr1 mutant DSY448 (Fig. 7).

The reason for the lack of hypersusceptibility of the Δcdr2 deletion mutant was clearer when the mRNA levels of this gene were measured in the parent strain CAF2-1 or in other azole-susceptible strains. In fact, no CDR2 mRNA was detected in these cells (Figs 4 and 8), even in the Δcdr1 mutant DSY447, which is the parent of the Δcdr1 Δcdr2 mutant DSY654 (data not shown). Taking these observations into account, it is expected that, when CDR2 is deleted in these strains, no phenotypic effect with respect to drug susceptibility can be observed.

**CDR2 is expressed in multidrug transporter mutants reverting spontaneously to normal fluconazole susceptibility**

We observed that, after a prolonged incubation of the Δcdr1 and Δcdr1 Δben deletion mutants DSY448 and DSY468 (which are described elsewhere; Sanglard et al., 1996) on plates containing fluconazole, some colonies spontaneously grew over a layer of spotted cells. The frequency at which these colonies appeared fluctuated between $1 \times 10^{-4}$ and $3 \times 10^{-4}$. Some of these cells were grown in drug-free YEPD medium and spotted again on plates containing fluconazole. To our surprise, five randomly chosen isolates, which were named R1–R5, were more resistant to fluconazole than their hypersusceptible parents. The same revertants regained resistance to ketoconazole and itraconazole (Table 3). This effect was not due to the expression of BENr, since Δcdr1 Δben mutants generated spontaneous revertants at the same frequency, and since overexpression of BENr can only render cells resistant to fluconazole. A Northern blot of RNA extracted from the revertant strains R1–R5 showed that CDR1 mRNA was, as expected, effectively absent from these cells, but that the CDR2 gene was expressed (Fig. 8). The level of this expression was low but significant in these cells when compared to the absence of expression in CAF2-1 or DSY447. Therefore it appears that the expression of CDR2 seems to be responsible for the reversion of R1–R5 to wild-type levels of susceptibility not only to fluconazole but also to ketoconazole and itraconazole. This result is not entirely unexpected given that CDR2 is clearly overexpressed in resistant strains (Fig. 4) and that it participates with CDR1 in the resistance of *C. albicans* to azole antifungal agents.
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Fig. 7. Susceptibility of multidrug transporter mutants DSY448, DSY653 and DSY654 to antifungal agents and metabolic inhibitors. Each strain was grown to exponential growth phase to a density of \(2 \times 10^7\) cells ml\(^{-1}\) and 5 \(\mu\)l was spotted in a dilution series on YEPD complex medium agar plates. Plates were incubated for 60 h at 30°C. The concentration of compounds utilized in this study is indicated for each plate.

Fig. 8. Northern blot analysis of C. albicans multidrug transporter mutants R1–R5 reverting to wild-type susceptibility to fluconazole. CAF4-2 and DSY468 were included in this analysis. Membranes were hybridized sequentially with the CDR1, CDR2 and TEF3 probes. The design of the CDR1 and CDR2 probes is described in the legend of Fig. 4. After hybridization with the CDR1 and CDR2 probes, membranes were exposed for 48 h on an RX Fuji X-ray film at -70°C. The exposure time for the TEF3 probe was 3 h.

Table 3. Multidrug transporter mutants reverting to wild-type levels of susceptibility to azole antifungal agents

<table>
<thead>
<tr>
<th>Strain type</th>
<th>Parent strain</th>
<th>YEPD only</th>
<th>Flu (0.5)</th>
<th>Keto (0.03)</th>
<th>Itra (0.0125)</th>
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<tr>
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<td>4</td>
<td>3</td>
<td>3</td>
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<tr>
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<td>1</td>
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<tr>
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<td>4</td>
<td>4</td>
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DISCUSSION

We report here the cloning of different C. albicans genes which conferred resistance to different antifungal agents, including azole derivatives, and to other unrelated metabolic inhibitors. Not only were the CDR1 and the BEN' genes re-isolated by a screening procedure consisting of complementing the fluconazole hypersusceptibility of an S. cerevisiae Δpdr5 mutant, but other additional genes were cloned using the same compound and with ketoconazole as a selective agent. Among these additional genes, a new ABC transporter gene closely related to CDR1 was characterized and therefore named CDR2. Prasad et al. (1995) utilized a similar strategy to clone multidrug resistance genes with a similar S. cerevisiae mutant but using cycloheximide as a selective agent in the screening procedure and two C. albicans genomic libraries based on single copy and multicopy vectors. Seventy-four different clones were isolated by these authors, which is much higher than in our case, although this result did not take into account the number of clones with overlapping restriction maps. The high number of clones obtained by Prasad et al. (1995) could have been due to the nature of the selective agent, which probably allows the cloning of genes whose action on drug resistance is much less specific than azole antifungal agents. Our preliminary data on the identity of the additional genes cloned in this study and which are different from CDR1, BEN' and CDR2 suggest that pDS255 and pDS257 contained genes similar to BEN' and to the S. cerevisiae transcription factor YAPI, respectively (D. Sanglard, unpublished).

The method of cloning resistance genes by functional complementation is of potential interest for obtaining similar genes from other pathogenic organisms. Candida glabrata and Candida krusei, for example, are organisms...
becoming more frequent in fungal infections and they exhibit lower intrinsic susceptibility to fluconazole than measured in \textit{C. albicans} (Essayag et al., 1996; Johnson et al., 1995a; Odds et al., 1996). Some reports document resistance to azole antifungal agents in \textit{C. glabrata} and \textit{C. krusei} after prolonged exposure to these agents (Berrouane et al., 1996; Chavanet et al., 1994; Hitchcock et al., 1993; Parkinson et al., 1995; Vanden Bossche et al., 1992), so understanding mechanisms of resistance in these isolates is becoming more important.

Table 2 gives an overview of the pattern of resistance of the cloned genes to different classes of antifungal agents and metabolic inhibitors. Besides the resistance conferred to three important azole derivatives by overexpression of \textit{CDR1} and \textit{CDR2} in \textit{S. cerevisiae}, it is noteworthy to mention the cross-resistance to other classes of antifungal agents such as terbinafine and amorolfin, which are used, as in the case of azole derivatives, in the treatment of different fungal infections. It is of interest that the expression of \textit{BEN} \textsuperscript{T} in the same host could make cells resistant to terbinafine and to a lesser extent to amorolfin. These features have an important impact for the treatment of fungal infections, since it is possible that resistance to azole antifungals mediated by the overexpression of multidrug transporters can extend to the other still-available antifungal agents.

The Cdr2p protein shares high similarity with Cdr1p, which in turn displays a high degree of similarity with the \textit{S. cerevisiae} Pdr5p (Balzi et al., 1994; Bissinger & Kuchler, 1994). The functional complementation of \textit{CDR1} and \textit{CDR2} in an \textit{S. cerevisiae} multidrug transporter mutant \textit{Apdr5} allows the two gene products to be distinguished from each other by the differences in pattern of resistance to various substances. The Cdr2p protein seems to have a lower potency than Cdr1p in the degree of resistance conferred to \textit{S. cerevisiae}, but gives a specific resistance to crystal violet. Interestingly, the \textit{S. cerevisiae} gene \textit{SGE1}, which encodes a protein belonging to the class of major facilitators, also confers specific resistance to crystal violet when overexpressed (Ehrenhofer et al., 1994). Whether or not the differences in resistance patterns between Cdr1p and Cdr2p are due to amino acid substitutions between the two proteins remains to be determined; for example, the features observed for the two proteins may still not be valid in the context of expression in \textit{C. albicans}, since the coding region of \textit{CDR2}, as opposed to \textit{CDR1}, contains three CUG codons (see Fig. 2) which are known to be translated to serine in this host and not to leucine as is the case in \textit{S. cerevisiae} (Santos & Tuite, 1995).

The most interesting feature of \textit{CDR2} at the moment is its regulation in \textit{C. albicans}. As judged by the absence of \textit{CDR2} mRNA signals after autoradiography of the Northern blot shown in Fig. 4, \textit{CDR2} was not expressed at levels detectable by this method in clinical \textit{C. albicans} strains susceptible to azole antifungal agents, but was overexpressed in isolates which became resistant to these agents. In a previous study (Sanglard et al., 1995), we showed that \textit{CDR1} was overexpressed in these strains. The probe for \textit{CDR1} generated at that time did not discriminate between \textit{CDR1} and \textit{CDR2} and thus the signal measured comprised the sum of the \textit{CDR1}- and \textit{CDR2}-specific signals. The elevated expression of \textit{CDR1} and \textit{CDR2} could be due to the action of the same or distinct transacting factors which remain to be characterized. The available sequence information on the promoters of both genes reveals no identical nucleotide regions and thus a common regulatory region cannot be assumed.

\textit{CDR2} expression can also be observed in multidrug transporter mutants when they revert spontaneously to wild-type levels of susceptibility to azole antifungal agents (Fig. 8; Table 3). The expression of \textit{CDR2} in these revertants can be considered a plausible explanation for this effect. Available data suggest that these revertants are even slightly more resistant than the wild-type parent CAF2-1 to azole derivatives (Table 3). Although we cannot exclude that other resistance factors may operate in these revertants, the restored \textit{CDR2} expression stresses the importance of this multidrug efflux transporter in the understanding of the mechanisms of resistance to azole antifungal agents in \textit{C. albicans}.

The disruption of the \textit{CDR2} gene in \textit{C. albicans} CAF4-2 did not result in hypersusceptibility to the substances tested in this study. Although \textit{CDR1} (which is present in this strain) may compensate for the deletion of \textit{CDR2}, it could also be argued that a residual Cdr2p activity might exist in the created homozygous \textit{Δcdr2} deletion mutant. It was not possible to measure the absence of \textit{CDR2} mRNA in the created mutant, since the wild-type parent already did not exhibit detectable \textit{CDR2} expression. However, the data available on the identity of the homozygous \textit{Δcdr2} deletion mutant allow us to ascertain a correct genotype. We believe that the lack of hypersusceptibility of the \textit{Δcdr2} mutant reflects the absence of \textit{CDR2} expression in CAF4-2.

Several lines of evidence now suggest that multidrug transporter genes of the class of ABC transporters may be members of a larger multigene family in \textit{C. albicans}. Besides the cloning of \textit{CDR1} and \textit{CDR2}, preliminary data from a screening of \textit{C. albicans} genomic library with a conserved multidrug ABC transporter gene probe resulted in the isolation of at least two distinct genes with similarity to the two genes mentioned above isolated by functional screening. It will be interesting to resolve the complexity of this gene family in the future and to determine if only specific members of this multigene family are involved in resistance to azole antifungal agents in \textit{C. albicans}.

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