

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, $\Delta pdr5$, 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 resistance*). 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 CAF4-2 did not render cells more susceptible to these substances. When the disruption of *CDR2* was performed in the background of a mutant in which *CDR1* was deleted, the resulting double $\Delta cdr1 \Delta cdr2$ mutant was more susceptible to these agents than the single $\Delta cdr1$ mutant. The absence of hypersusceptibility of the single $\Delta cdr2$ 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 $\Delta cdr1$ 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

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.

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),

which is a haemoprotein involved in the 14 α -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 Benp, 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 Pdr5p/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 Δ *pdr5* resistant to these agents was developed. The possible involvement of cloned genes in the resistance to azole 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 (Δ *ura3::imm434*/ Δ *ura3::imm434*) and the parent CAF2-1 (Δ *ura3::imm434*/*URA3*) were 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 sts1::TRP1*) has been described by Bissinger & Kuchler (1994). The genotypes of other yeast strains are listed in Table 1. *E. coli* DH5 α (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 μ g 5-fluoroorotic acid (5-FOA) ml⁻¹ were made for the regeneration of the *ura3* genetic marker in YNB selective medium with 50 μ g 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), amorolfine 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 \times 10⁷ cells ml⁻¹ in 0.9% NaCl. Five microlitres of this suspension and of serial dilutions of each yeast culture was 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 2.4 kb *SpeI*-*KpnI* fragment from pDS246 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 pMB-7 (Fonzi & Irwin, 1993) to create pDS368. The linear 5.2 kb *SpeI*-*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 \times 10⁷ 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 μ l Li/TE buffer and placed on ice. Yeast suspension (50 μ l) was aliquoted into Eppendorf tubes. The following reagents were added sequentially to each tube: 300 μ l of freshly prepared PEG buffer (40% PEG 4000 in Li/TE buffer), 5 μ l 10 mg sheared herring sperm DNA (Clontech) ml⁻¹, then linear DNA fragments (1–2 μ g) in a maximum volume of 5 μ l. 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.

Table 1. Genotypes of yeast strains used in this study

Strain name	Parent strain	Genotype	Reference
CAF2-1	SC5314	$\Delta ura3::imm434/URA3$	Fonzi & Irwin (1993)
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)
DSY449	DSY448	$\Delta cdr1::hisG/\Delta cdr1::hisG$	Sanglard <i>et al.</i> (1996)
DSY649	CAF4-2	$\Delta cdr2::hisG-URA3-hisG/CDR2$	This study
DSY651	DSY649	$\Delta cdr2::hisG/CDR2$	This study
DSY653	DSY651	$\Delta cdr2::hisG-URA3-hisG/\Delta cdr2::hisG$	This study
DSY650	DSY449	$\Delta cdr1::hisG/\Delta cdr1::hisG$	This study
		$\Delta cdr2::hisG-URA3-hisG/CDR2$	
DSY652	DSY650	$\Delta cdr1::hisG/\Delta cdr1::hisG$	This study
		$\Delta cdr2::hisG/CDR2$	
DSY654	DSY652	$\Delta cdr1::hisG/\Delta cdr1::hisG$	This study
		$\Delta cdr2::hisG-URA3-hisG/\Delta cdr2::hisG$	

The supernatant was removed and the cell pellet was resuspended in 200 μ l 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 μ l) was electroporated into *E. coli* DH5 α 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 \times SSC, 10% dextran sulfate and 100 μ g salmon sperm DNA ml⁻¹. [³²P]DNA-labelled probes were generated by random priming (Feinberg & 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 μ l 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 μ l phenol/chloroform/isoamyl alcohol (24:24:1, by vol.). 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 μ l TE. Of this suspension, 1 μ l was used for PCR.

RESULTS

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

We reported recently that an *S. cerevisiae* mutant YKKB-13 lacking the ABC transporter Pdr5p was hypersusceptible to azole 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 to azole 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 YKKB-13. A total of 40000 Ura⁺ clones was obtained and spotted in selective YNB plates containing in a first screening 10 μ g fluconazole ml⁻¹. Thirty-two azole-resistant clones emerged and their plasmids were

Table 2. At least six *C. albicans* genes confer resistance to azole antifungal agents and other metabolic inhibitors in *S. cerevisiae*

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 10^{-3} ; 2, growth at 10^{-2} ; 1, growth at 10^{-1} ; 0, growth at 10^{-0} ; —, no growth at 10^{-0} .

Substance*	Plasmid						
	YE24	pDS243	pDS239	pDS246	pDS255	pDS257	pDS270†
YEPD only	4	4	4	4	4	4	4
Flu (5)	—	4	4	2	4	2	1
Itra (0.025)	—	4	—	2	—	—	1
Keto (0.05)	—	3	—	3	—	—	1
Cyh (0.025)	—	4	4	2	3	2	—
NQO (50)	—	—	4	—	—	—	—
Ben (50)	—	—	4	—	—	—	—
Terb (8)	0	4	3	2	1	1	1
Amo (0.05)	—	3	0	1	—	—	—
Sum (50)	0	3	4	0	2	0	0
Flup (10)	—	4	2	3	—	—	—
Phe (10)	—	—	4	—	—	—	—
Rho (5)	—	4	—	2	—	—	1
Cer (0.5)	0	4	4	4	2	4	0
Bref (25)	2	4	4	4	4	4	2
Cry (0.05)	—	0	—	2	—	—	—

* Abbreviations for substances are followed by a number giving the concentration in $\mu\text{g ml}^{-1}$ 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, sulfomethuron methyl; Terb, terbinafine.

† pDS270 was isolated by ketoconazole-resistance selection.

rescued in *E. coli* DH5 α 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 to azole 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, pDS257 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 three azole 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 *BEN^r* genes (Sanglard *et al.*, 1995). *CDR1* had previously been cloned by Prasad *et al.* (1995) by complementation of cycloheximide hypersusceptibility of an *S. cerevisiae* Δpdr5 mutant, while *BEN^r* 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), sulfomethuron methyl, rhodamine 6G, cerulenin and brefeldin A. These compounds have not yet been reported as substrates for this transporter. pDS239, which contained *BEN^r*, 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, sulfomethuron methyl, 1,10-phenanthroline, cerulenin and brefeldin A (Table 2). Among these new substrates, terbinafine, sulfomethuron methyl, cerulenin and brefeldin A can be used as substrates by both multidrug transporter classes. Specific substrates for Benp are

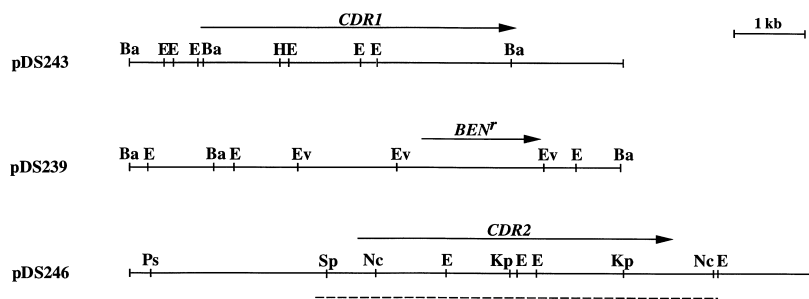


Fig. 1. Restriction maps of *C. albicans* insert DNA from plasmids conferring resistance to fluconazole in the *S. cerevisiae* $\Delta pdr5$ mutant YKKB-13 (see Table 2). Only maps of pDS243, pDS239 and pDS246 are shown here. Ba, *Bam*HI; E, *Eco*RI; Ev, *Eco*RV; H, *Hind*III; Nc, *Nco*I; Kp, *Kpn*I; Ps, *Pst*I; Sp, *Spe*I. Plasmids pDS243 and pDS239 contained the *CDR1* and *BEN^r* genes, whose restriction maps are identical to those reported by Prasad *et al.* (1995) and by Fling *et al.* (1991), respectively. pDS246 contained the *CDR2* gene. The *CDR2* sequenced segment (Fig. 2) is indicated by a dotted line. Positions and transcription directions of the ORF of each gene are shown by arrows.

benomyl, 1,10-phenanthroline and 4-nitroquinoline *N*-oxide.

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 *Nla*IV–*Eco*RI 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 adenosine 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 chromosome 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 hydropathy plot of *Cdr2p* identified six putative transmembrane 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 (IQCV) 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 to azole 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


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Cdr1p 1 . . M S D S K M S S Q . D E S K L E K A I S Q D S S E M S H N E Y H G F D A H T S E N I Q N L A R T F T H D S F K D 57
      . . . . . | . . . . . | . . . . . | . . . . . | . . . . . | . . . . . | . . . . . | . . . . . | . . . . . | . . . . . |
Cdr2p 1 M S T A N T S L S Q Q L D E N P W D A L . . . . . D N S S V Q E Y Q G F D A T A S H N I Q D L A R K L T H G S T N G 54
      | . . . . . | . . . . . | . . . . . | . . . . . | . . . . . | . . . . . | . . . . . | . . . . . | . . . . . |
58 D . . S S A G L L K Y L T H M S E V G V N P Y E H E E I N N D Q L N P D S E N F N A K F W K N L R K L F E S D P E Y 115
      | . . . . . | . . . . . | . . . . . | . . . . . | . . . . . | . . . . . | . . . . . | . . . . . | . . . . . |
55 D H H S A N D L A R Y L S H M S D I P G V S P F . N G N I S H E Q L D P D S E N F N A K Y W K N L K L F E S D S D Y 113
      | . . . . . | . . . . . | . . . . . | . . . . . | . . . . . | . . . . . | . . . . . | . . . . . | . . . . . |
116 Y K P S K L G I G Y R N L R A Y G V A N D S D Y Q P T V T N A L W K L A T E G F R H F Q K D D S R Y F D I L K S M D A 175
      | . . . . . | . . . . . | . . . . . | . . . . . | . . . . . | . . . . . | . . . . . | . . . . . | . . . . . |
114 Y K P S K L G V A Y R N L R A Y G I A N D S D Y Q P T V T N A L W K F T P E A I N K L K P D D S K Y F D I L K S M D A 173
      | . . . . . | . . . . . | . . . . . | . . . . . | . . . . . | . . . . . | . . . . . | . . . . . | . . . . . |
176 I M R P G E L T V V I G R P G A C C S T L L K T I A V N T Y G F H I G K E S Q I T Y D G L S P H D I E R H Y R G D V I Y 235
      | . . . . . | . . . . . | . . . . . | . . . . . | . . . . . | . . . . . | . . . . . | . . . . . | . . . . . |
174 I M R P G E L T V V I G R P G A C C S T L L K T I A V N T Y G F H I G K E S Q I T Y D G L S P H D I E R H Y R G D V I Y 233
      | . . . . . | . . . . . | . . . . . | . . . . . | . . . . . | . . . . . | . . . . . | . . . . . | . . . . . |
236 S A E T D V H F P H L S V G D T L E F A A R L R T P Q N R G E G I D R E T Y A K H M A S V Y M A T Y G L S H T R N T N V 295
      | . . . . . | . . . . . | . . . . . | . . . . . | . . . . . | . . . . . | . . . . . | . . . . . | . . . . . |
234 S A E T D V H F P H L S V G D T L E F A A R L R T P Q N R G E G I D R E T Y A K H M A S V Y M A T Y G L S H T R N T N V 293
      | . . . . . | . . . . . | . . . . . | . . . . . | . . . . . | . . . . . | . . . . . | . . . . . | . . . . . |
296 G N D F V R G V S G G E R K R V S I A E A S L S G A N I Q C W N A T R G L D S A T A L E F I R A L K T S A V I L D T T 355
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294 G N D F V R G V S G G E R K R V S I A E A S L S G A N I Q C W N A T R G L D S A T A L E F I R A L K T S A T I L D T T 353
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356 P L I A I Y Q C S Q D A Y L D F K V V V L Y E G Y I F G K A T K A K E Y P E K M G W K C P O R Q T T A D F L T S L 415
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354 P L I A I Y Q C S Q D A Y L E F D N V V V L Y E G Y I F G K A S K A K E Y P E N M G W K C P O R Q T T A D F L T S L 413
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416 T N P A E R E P L P G Y E D K V P R T A Q E F E T Y W K N S P E Y A E L T K E I D E Y F V E C E R S N T R E T Y R E S H 475
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414 T N P A E R E P L P G Y E D K V P R T A Q E F E T F W N K S P E Y A E L T K E I D E Y F V E C E R S N T G E T Y R E S H 473
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476 V A Q S N N T R P A S P Y T V S F F Q V R Y G V A R N F L R M K G D P S I P I P S V F G Q L V M G L L S S V F Y N 535
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474 V G K S N N T R P S S P Y T V S F F Q V R V I A R N F L R M K G D P S I P L I S I L S Q L V M G L I A S V F F N 533
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536 L S Q T T G S F Y R G A A M F A V L F N A F S S L L E I M S L F E A R P I V E K H K K Y A L Y R P S A D A L A S I I 595
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534 L R K S T D T F P R G A L F F S V L F N A F S S L L E I L S L Y E A R P I V E K H R K Y A L Y R P S A D A L A S I I 593
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594 S E L P V K L M T M S F N I V Y P M W N L R R P A G N F F Y M L C A L C T L V M S H M F R S I G A V T T I A T 653
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656 A M T P A T V L L L A M V I Y T G F V I P T P S M L G W S R W I N Y I N P V G V P E S L M V N E P H G R E F Q C A O Y 715
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654 A M S L S T V P L L A M I Y A G F V L P I P Y I L G W S R W I R Y I N P V T Y I P E S L M V N E P H G R E F E C G O Y 713
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716 V P S G P G Y E N I S R S N Q V C T A V G S V P G N E M V S G I N Y L A G A Y Q Y N S H K W R N L G I T I G F A V F F 775
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714 I P S G P G F E N L P V E N K V C T T V G S T P G S T V V Q G T E Y I K L A Y Q F Y S H K W R N F G I T V A F A V F F 773
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776 L A I Y I A L T E P N K G A M Q G E I V L F L K G S L K K H R K T A A S N K G D I E A G P V A G K L D Y Q D E A E A 835
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774 L G V V A L T E P N K G A L Q G E I V L F L K G S L K K H R K T A A S N K G D I E A G P V A G K L D Y Q D E A E A 833
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836 V N N E K F T E K G S T G S V D F P E N R E I F F W R D L T Y Q V K I K K E D R V I L D H V D G W K P G Q I T A L M G 895
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834 V N N E K F T E K G S T G S V D F P E N R E I F F W R D L T Y Q V K I K K E D R V I L D H V D G W K P G Q I T A L M G 893
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896 A S G A G K T T L L N C L S E R V T T G I I T D G E R L V N G H A L D S S F O R S I G Y V Q O Q D V H L P T S T V R E A 955
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894 A S G A G K T T L L N C L S E R V T T G I I T D G E R L V N G H A L D S S F O R S I G Y V Q O Q D V H L E T T V R E A 953
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956 L Q F S A Y L R Q S N K I S K K E K D D Y V D V I D L L E M T D Y A D A L V G V A G E G L N V E Q R K R L T I G V E L 1015
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954 L Q F S A Y L R Q S N K I S K K E K D D Y V D V I D L L E M T D Y A D A L V G V A G E G L N V E Q R K R L T I G V E L 1013
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1016 V A K P K L L L F L D E P T S G L D S Q T A M S I C K L M R K L A D H G Q A I L C T I H Q P S A L I M A E F D R L L F L 1075
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1014 V A K P K L L L F L D E P T S G L D S Q T A M S I C K L M R K L A D H G Q A I L C T I H Q P S A L I M A E F D K L L F L 1073
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1076 Q K G R T A Y F G E L G E N C Q T M I N Y F E K Y G A D P C P K E A N P A E W M L Q V G A A P G S H A K Q D Y F E V 1135
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1074 Q K G R T A Y F G E L G E N C Q T M I N Y F E K Y G A D P C P K E A N P A E W M L Q V G A A P G S H A K Q D Y F E V 1133
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1136 W R N S S E Y Q A V R E E I N R M E A E L S K L P R D N P E A L L K Y A A P L W K Q Y L L V S W R T I V Q D W R S P G 1195
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1134 W R N S S E Y Q A V R E E I N R M E A E L S K L P R D N P E A L L K Y A A P L W K Q Y L L V S W R T I V Q D W R S P G 1193
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1196 Y I Y S K I F L V V S A A L F N G F S P F K A K N M G L Q N Q M F S V F M F I P F N T L V Q Q M L P Y F V K Q R D 1255
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1194 Y I Y S K L I V I S S S L F I G F S P F K S K N L Q L Q S Q M L A V F M F V P P T T F I D Q M L P Y F V K H R A 1253
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1256 V Y E V R E A P S R T F S W F A F I A G Q I T S E I P Y Q V A V G T I A F F C W Y I P L G L Y N A T P T D S V N P R G 1315
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1254 V Y E V R E A P S R T F S W F A F I A G Q I T S E I P F Q I V G T I S Y F C W Y I P V G L Y A N A E P T D S V N S R G 1313
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1316 V L M W M L V T A P Y V Y T A T M Q C M S F S E L A D N A A N L A T L L F T M C L N F C G V L A G P D V L P G F W I 1375
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1314 V L M W M L L T A P Y V Y T S T M Q L A I S I N L E I D N A A N L A T L L F T L C L M F C G V L A G P N V I P G F W I 1373
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1376 F M Y R C N P F T Y L V Q A M L S T G L A N T F V K A E R E Y V S V K P P N G E S C S T Y L D P Y I K F A G G Y F E T 1435
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1374 F M Y R C N P F T Y L Q A I L S T G L A N A K V T C A P E R I V L T K P P M G E T C S S P I G P Y E A A G G Y F S T 1433
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1436 R N D G S A C F Q M S S T N F L K S V N S L Y S E R W R N F G I F I A I N I I L T V I P Y W L A R V P K G N R 1495
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1434 N S D G T C S V C R I D S T N Q F L E S I N A L F S Q R W R N F G I F V A F I G I N I I L T I F F Y W L A R V P K G N R 1493
      | . . . . . | . . . . . | . . . . . | . . . . . | . . . . . | . . . . . | . . . . . | . . . . . | . . . . . |
1496 E K I N K K 1501
      | . . . . . |
1494 E K I M K K 1499
      | . . . . . |
    
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Fig. 3. Alignment of the *C. albicans* multidrug resistance proteins Cdr1p and Cdr2p. Identical amino acid residues are

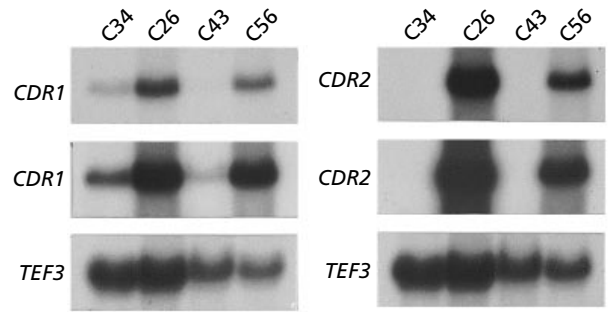


Fig. 4. Northern blot analysis of *C. albicans* clinical isolates susceptible and resistant to azole antifungal agents. Isolates C34 and C26, and C43 and C56 are azole-susceptible and azole-resistant pairs from two distinct patients. These strains have been characterized and have been tested for their 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* probes 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-URA3-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 would anchor a site flanking the region of homologous recombination between the genomic locus and the linear DNA fragment (see Fig. 5). Ten Ura⁺ transformants for each disruption in CAF4-2 and DSY449 were screened and they all showed the correct genotype. PCR analysis of only one of these clones for each disruption strategy

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. The alignment was generated by the method of Needleman & Wunsch (1970) implemented in the program GAP of the GCG package of the University of Wisconsin.

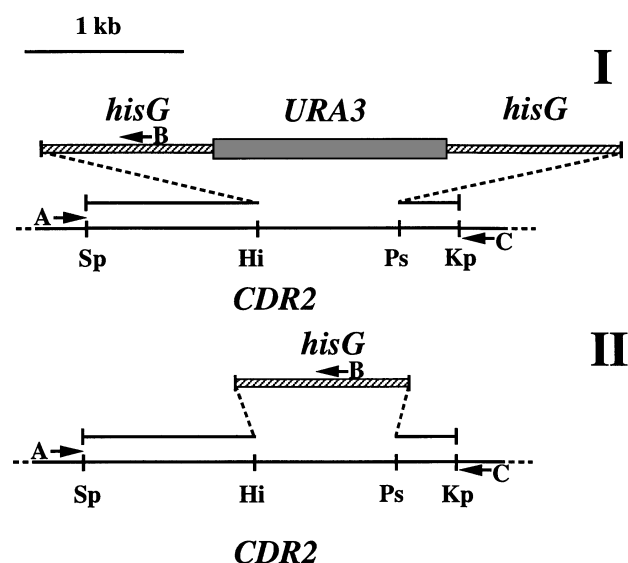


Fig. 5. Restriction maps of the *CDR2* alleles in disruption experiments. Parts I and II are schematic representations of the disruption of the *CDR2* gene before and after regeneration of the *ura3* genetic marker by selection with 5-FOA. The maps of the wild-type alleles are shown with the corresponding disrupted alleles. The location of the primers A (5'-ATTCAATTCACGGAAATCGGATA-3'; *CDR2*-specific), B (5'-GGCGCGACTTCGACAGAAC-3'; *hisG*-specific) and C (5'-GTGCACAGTGCACACATTAACC-3'; *CDR2*-specific) used in PCR for the monitoring of disruption (Fig. 6) is 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 on 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 $\Delta cdr2::hisG$ 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*⁺ $\Delta cdr2$ and $\Delta cdr1 \Delta cdr2$ 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 $\Delta cdr1$ 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 $\Delta cdr2::hisG$ 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 $\Delta cdr2$ homozygote deletion mutants created were then used in drug susceptibility assays.

Drug susceptibility assays with $\Delta cdr2$ and $\Delta cdr1 \Delta cdr2$ 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 substance due to the absence of its specific efflux transporter. The previously constructed $\Delta cdr1$ 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 $\Delta cdr2$ 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 $\Delta cdr1$ background can a more severe growth inhibition be observed compared to the single $\Delta cdr1$ 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 $\Delta cdr1$ and the double $\Delta cdr1 \Delta cdr2$ mutants. Intracellular concentrations of [³H]fluconazole were measured in these mutants to illustrate these observations, as indicated in Methods. Whereas the $\Delta cdr2$ deletion mutant DSY653 accumulated approximately the same levels of fluconazole as the wild-type (670 ± 25 and 718 ± 21 c.p.m. per 10^9 cells, respectively), accumulation in the $\Delta cdr1$ and the double $\Delta cdr1 \Delta cdr2$ mutants was increased by a factor of 2.3 and 3.1. These data are consistent with an increased susceptibility to fluconazole

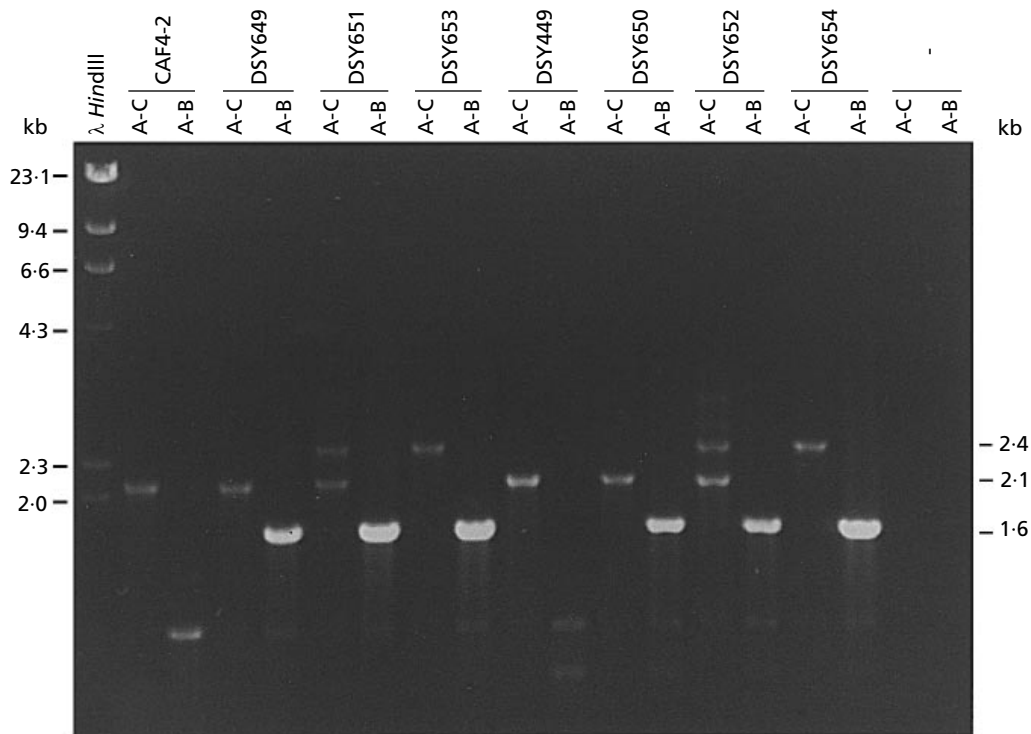


Fig. 6. PCR monitoring of the disruption of the *CDR2* gene in CAF4-2 and in the $\Delta cdr1$ deletion mutant DSY449. The sequences of the primers A, B and C are given in Fig. 5. The expected product size for amplifications with primers A and B is 1.6 kb on a disrupted allele whereas no amplification should be obtained with the wild-type allele. The bands seen at 1 kb and sometimes at 0.8 kb using primers A and B and DNA from CAF4-2 and other isolates represent a background. Expected product sizes with primers A and C are 2.1 and 2.4 kb for the wild-type allele and the disrupted allele after 5-FOA treatment. These expected PCR product sizes correspond to those observed by horizontal gel electrophoresis. The origin of genomic DNA from each strain is indicated with the corresponding primers used for each PCR. Yeast genotypes are given in Table 1.

of the $\Delta cdr1 \Delta cdr2$ mutant DSY654 compared to the $\Delta cdr1$ mutant DSY448 (Fig. 7).

The reason for the lack of hypersusceptibility of the $\Delta cdr2$ 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 $\Delta cdr1$ mutant DSY447, which is the parent of the $\Delta cdr1 \Delta cdr2$ 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 $\Delta cdr1$ and $\Delta cdr1 \Delta ben$ 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 1×10^{-4} and 3×10^{-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 *BEN^r*, since $\Delta cdr1 \Delta ben$ mutants generated spontaneous revertants at the same frequency, and since overexpression of *BEN^r* 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 itraconazole and ketoconazole. 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.

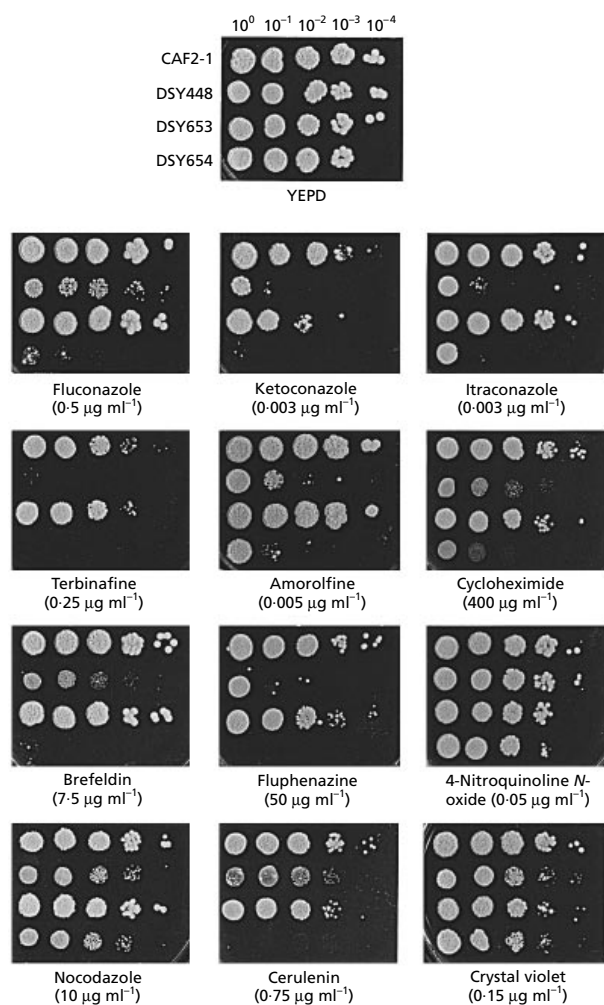


Fig. 7. Susceptibility of multidrug transporter mutants DSY448, DSY653 and DSY654 to antifungal agents and metabolic inhibitors. Each strain was grown to exponential growth phase to a density of 2×10^7 cells ml^{-1} and 5 μl was spotted in a dilution series on YEPD complex medium agar plates. Plates were incubated for 60 h at 30 °C. The concentration of compounds utilized in this study is indicated for each plate.

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^r* 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

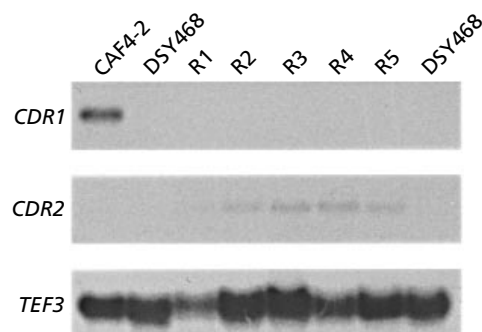


Fig. 8. Northern blot analysis of *C. albicans* multidrug transporter mutants R1–R5 reverting to wild-type susceptibility to fluconazole. CAF4-2 and DSY468 were included in this analysis. Membranes were hybridized sequentially with the *CDR1*, *CDR2* and *TEF3* probes. The design of the *CDR1* and *CDR2* 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

See Table 2 for explanation of values and abbreviations.

Strain type	Parent strain	YEPD only	Flu (0.5)	Keto (0.03)	Itra (0.0125)
CAF2-1	SC5314	4	4	3	3
DSY448	DSY447	4	2	0	1
DSY468	DSY467	4	2	0	1
R1	DSY468	4	4	4	4
R2	DSY448	4	4	4	4
R3	DSY468	4	4	4	4
R4	DSY468	4	4	4	4
R5	DSY448	4	4	4	4

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^r* and *CDR2* suggest that pDS255 and pDS257 contained genes similar to *BEN^r* and to the *S. cerevisiae* transcription factor *YAP1*, 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 *C. albicans* (Essayag *et al.*, 1996; Johnson *et al.*, 1995a; Odds *et al.*, 1996). Some reports document resistance to azole antifungal agents in *C. glabrata* and *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 *CDR1* and *CDR2* in *S. cerevisiae*, it is noteworthy to mention the cross-resistance to other classes of antifungal agents such as terbinafine and amorolfine, 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 *BEN*^r in the same host could make cells resistant to terbinafine and to a lesser extent to amorolfine. 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 *S. cerevisiae* Pdr5p (Balzi *et al.*, 1994; Bissinger & Kuchler, 1994). The functional complementation of *CDR1* and *CDR2* in an *S. cerevisiae* multidrug transporter mutant $\Delta pdr5$ 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 *S. cerevisiae*, but gives a specific resistance to crystal violet. Interestingly, the *S. cerevisiae* gene *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 *C. albicans*, since the coding region of *CDR2*, as opposed to *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 *S. cerevisiae* (Santos & Tuite, 1995).

The most interesting feature of *CDR2* at the moment is its regulation in *C. albicans*. As judged by the absence of *CDR2* mRNA signals after autoradiography of the Northern blot shown in Fig. 4, *CDR2* was not expressed at levels detectable by this method in clinical *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 *CDR1* was overexpressed in these

strains. The probe for *CDR1* generated at that time did not discriminate between *CDR1* and *CDR2* and thus the signal measured comprised the sum of the *CDR1*- and *CDR2*-specific signals. The elevated expression of *CDR1* and *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.

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 *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 *CDR2* expression stresses the importance of this multidrug efflux transporter in the understanding of the mechanisms of resistance to azole antifungal agents in *C. albicans*.

The disruption of the *CDR2* gene in *C. albicans* CAF4-2 did not result in hypersusceptibility to the substances tested in this study. Although *CDR1* (which is present in this strain) may compensate for the deletion of *CDR2*, it could also be argued that a residual Cdr2p activity might exist in the created homozygous $\Delta cdr2$ deletion mutant. It was not possible to measure the absence of *CDR2* mRNA in the created mutant, since the wild-type parent already did not exhibit detectable *CDR2* expression. However, the data available on the identity of the homozygous $\Delta cdr2$ deletion mutant allow us to ascertain a correct genotype. We believe that the lack of hypersusceptibility of the $\Delta cdr2$ mutant reflects the absence of *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 *C. albicans*. Besides the cloning of *CDR1* and *CDR2*, preliminary data from a screening of a *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 *C. albicans*.

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