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Susceptibilities of *Candida albicans* Multidrug Transporter Mutants to Various Antifungal Agents and Other Metabolic Inhibitors

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Some *Candida albicans* isolates from AIDS patients with oropharyngeal candidiasis are becoming resistant to the azole antifungal agent fluconazole after prolonged treatment with this compound. Most of the *C. albicans* isolates resistant to fluconazole fail to accumulate this antifungal agent, and this has been considered a cause of resistance. This phenomenon was shown to be linked to an increase in the amounts of mRNA of a *C. albicans* ABC (ATP-binding cassette) transporter gene called *CDR1* and of a gene conferring benomyl resistance (*BEN*^{r}), the product of which belongs to the class of major facilitator multidrug efflux transporters (D. Sanglard, K. Kuchler, F. Ischer, J. L. Pagani, M. Monod, and J. Bille, Antimicrob. Agents Chemother. 39:2378–2386, 1995). To analyze the roles of these multidrug transporters in the efflux of azole antifungal agents, we constructed *C. albicans* mutants with single and double deletion mutations of the corresponding genes. The mutants were tested for their susceptibilities to these antifungal agents. Our results indicated that the Δ*cdr1 C. albicans* mutant was hypersusceptible to the azole derivatives fluconazole, itraconazole, and ketoconazole, thus showing that the ABC transporter Cdr1 can use these compounds as substrates. The Δ*cdr1* mutant was also hypersusceptible to other antifungal agents (terbinafine and amorolfine) and to different metabolic inhibitors (cycloheximide, brefeldin A, and fluphenazine). The same mutant was slightly more susceptible than the wild type to nocodazole, cerulenin, and crystal violet but not to amphotericin B, nikkomycin Z, flucytosine, or pradimicin. In contrast, the Δ*ben* mutant was rendered more susceptible only to the mutagen 4-nitroquinoline-*N*-oxide. However, this mutation increased the susceptibilities of the cells to cycloheximide and cerulenin when the mutation was constructed in a Δ*cdr1* background. The assay used in the present study could be implemented with new antifungal agents and is a powerful tool for assigning these substances as putative substrates of multidrug transporters.

Candida albicans is an opportunistic pathogen important in humans and the major agent of oropharyngeal candidiasis in AIDS patients (23). Among the antifungal agents available for treating infections caused by *Candida* spp., fluconazole is by far the most commonly used compound (24). The cellular target of fluconazole and other azole derivatives in *C. albicans* is a cytochrome P-450, which is a hemoprotein involved in the 14α-demethylation of lanosterol, an important step in the biosynthesis of ergosterol (28). As a consequence of the increasing number of infections caused by *C. albicans* in AIDS patients, the use of this antifungal agent has also been more frequent. Repeated treatments with fluconazole have led to the appearance of yeast isolates resistant in vitro to this agent (17, 26, 29). 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 enhanced fluconazole efflux. Two multidrug efflux transporters, the ABC (ATP-binding cassette) transporter Cdr1 and the major facilitator Ben^r were identified as possible mediators in this process (27). The genes for these transporters, *CDR1* and *BEN*^{r}, have been isolated by Prasad et al. (25) and Fling et al. (8) and also in our laboratory by complementation of fluconazole hypersusceptibility of a *Saccharomyces cerevisiae* mutant lacking the ABC transporter Pdr5/Sts1 (27).

Now that a genetic system has become available for *C. albicans* (9), the involvement of these multidrug transporters in the efflux of azole antifungal agents could be tested by constructing mutants lacking these transporters and by subjecting them to susceptibility tests. Here we report the construction of these mutants and their susceptibilities to different azole antifungal agents. To show that multidrug transporters can accept a large variety of different substrates, the susceptibility tests were extended to other medically important antifungal agents and different metabolic inhibitors.

MATERIALS AND METHODS

Strains. *C. albicans* CAF4-2 (Δ*ura3*::*imm434*/Δ*ura3*::*imm434*) and the parent CAF2-1 (Δ*ura3*::*imm434*/*URA3*) were received from B. Fonzi (9). The genotypes of the other yeast strains tested are listed in Table 1. *Escherichia coli* DH5α (16) was used as a host for plasmid construction and propagation.

Media. The *C. albicans* strains were grown on YEPD complex medium, which contained 2% glucose, 1% Bacto Peptone (Difco Laboratories, Detroit, Mich.), 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 the transformation of *C. albicans*. Agar plates containing 50 μg of 5-fluoroorotic acid (5-FOA) per ml were made for the regeneration of the *ura3* genetic marker in YNB selective medium with 50 μg of uridine per ml.

Construction of plasmids. For the disruption of *CDR1* in *C. albicans*, a 2.8-kb *Eco*RV-*Kpn*I fragment from pDS243, a plasmid described previously (27), was first subcloned into the same sites of pBluescriptKS+ (Stratagene, La Jolla, Calif.), thus yielding pDS278. An internal 1.4-kb *Hin*dIII-*Bgl*II fragment from pDS278 was replaced by a 3.7-kb *Hin*dIII-*Bgl*II fragment from pMB-7 (9), which

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TABLE 1. Genotypes of strains used in the study

Strain name	Parent strain	Genotype	Reference or source
CAF2-1	SC5314	Δ*ura3*::*imm434*/*URA3*	9
CAF4-2	CAF2-1	Δ*ura3*::*imm434*/Δ*ura3*::*imm434*	9
DSY446	CAF4-2	Δ*cdr1*::*hisG-URA3-hisG*/*CDR1*	This study
DSY447	DSY446	Δ*cdr1*::*hisG*/*CDR1*	This study
DSY448	DSY447	Δ*cdr1*::*hisG-URA3-hisG*/Δ*cdr1*::*hisG*	This study
DSY449	DSY448	Δ*cdr1*::*hisG*/Δ*cdr1*::*hisG*	This study
DSY463	CAF4-2	Δ*ben*::*hisG-URA3-hisG*/*BEN*	This study
DSY464	DSY463	Δ*ben*::*hisG*/*BEN*	This study
DSY465	DSY464	Δ*ben*::*hisG-URA3-hisG*/Δ*ben*::*hisG*	This study
DSY466	DSY449	Δ*cdr1*::*hisG*/Δ*cdr1*::*hisG*Δ*ben*::*hisG-URA3-hisG*/*BEN*	This study
DSY467	DSY466	Δ*cdr1*::*hisG*/Δ*cdr1*::*hisG*Δ*ben*::*hisG*/*BEN*	This study
DSY468	DSY467	Δ*cdr1*::*hisG*/Δ*cdr1*::*hisG*Δ*ben*::*hisG-URA3-hisG*/Δ*ben*::*hisG*	This study

contained the *URA3* gene flanked by *Salmonella typhimurium hisG* repeats. This plasmid was named pDS279. A linear fragment containing the gene deletion construct was obtained by digestion of pDS279 by *Apa*I and *Kpn*I.

For the disruption of *BEN*^{r}, a *Pvu*II-*Bam*HI fragment of 4.5 kb from pDS245 (27) was subcloned into pBluescriptKS+ previously restricted by *Hin*dII and *Bam*HI, thus yielding pDS286. An internal *Sal*I-*Bgl*II fragment was replaced by a 3.7-kb fragment from pMB-7 as mentioned above to obtain pDS287. A linear fragment containing the gene deletion construct was obtained by digestion of pDS287 with *Spe*I and *Xho*I, which both have sites in the polylinker of pBluescriptKS+.

Accumulation of labelled azole derivatives in *C. albicans*. Experiments of [^{3}H]fluconazole (Amersham) accumulation in *C. albicans* CAF2-1 and in multidrug transporter mutants were performed as indicated previously (27), except that 20 min of incubation was chosen as a single sampling time.

Yeast transformation. For gene disruptions, *C. albicans* CAF4-2 was transformed with linear fragments by a lithium acetate procedure developed in our laboratory. The *C. albicans* isolates were first grown to the mid-logarithmic phase to a density of 2×10^7 cells per ml in 100 ml of YEPD complex medium with constant shaking at 250 rpm. A total of 50 ml of this culture was centrifuged in a 50-ml Falcon tube at 5,300 × *g* at 4°C for 5 min and was washed twice with TE buffer (10 mM Tris-HCl [pH 7.5], 1 mM EDTA). Another washing was performed with 10 ml of Li-TE buffer (0.1 M lithium acetate in TE [pH 7.5]). The final cell pellet was resuspended in 200 μl of Li-TE buffer and left on ice. A total of 50 μl of the yeast suspension was divided into aliquots and placed in Eppendorf tubes. The following reagents were added sequentially to each tube: 300 μl of freshly prepared polyethylene glycol (PEG) buffer (40% PEG 4000 in Li-TE buffer), 5 μl of 10 μg of sheared herring sperm DNA (Clontech Laboratories Inc., Palo Alto, Calif.) per μl, and then linear DNA fragments (1 to 2 μg) in a maximum volume of 5 μl. The contents of the tubes were mixed gently, and the tubes were incubated for 30 min at 30°C. After heat shock at 42°C for 20 min, the tubes were centrifuged three times by short spins in a microcentrifuge at room temperature. The supernatant was removed, and the cell pellet was resuspended in 200 μl of TE buffer. Aliquots of this suspension were plated onto YNB selective medium and the plates were incubated for 3 to 4 days at 30°C.

PCR. PCR buffers and *AmpliTaq* polymerase were from Perkin-Elmer (Roche Molecular Systems, Branchburg, N.J.). The buffer composition was 10 mM Tris-HCl (pH 8.3), 50 mM KCl, and 1.5 mM $MgCl_2$ containing 0.2 mM (each) deoxynucleoside triphosphates and 2.5 U of polymerase per reaction mixture. Briefly, PCR was carried out in a Thermal Cycler 480 (Perkin-Elmer) with a first cycle of denaturation at 94°C for 4 min and then 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. The PCR was completed by a final elongation step at 72°C for 10 min. The primers for PCR are described in Fig. 2 and 3 and were designed on the basis of the *C. albicans CDR1*, *C. albicans BEN*^{r}, and *S. typhimurium hisG* nucleotide sequences accessible in GenBank.

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

Drug susceptibility testing. The susceptibilities of the *C. albicans* strains to different compounds were tested qualitatively by spotting serial dilutions of yeast cultures in agar plates containing YEPD complex medium, which provides an easy means of visualizing the growth differences between different yeast strains. The following drugs were dissolved in dimethyl sulfoxide: ketoconazole and itraconazole (Janssen Pharmaceuticals, Beerse, Belgium), terbinafine (Sandoz Pharma Ltd., Basel, Switzerland), and nocodazole, brefeldin A, cerulenin, and 4-nitroquinoline-*N*-oxide (NQO; Sigma). Fluconazole (Pfizer UK, Sandwich, United Kingdom), amorolfine chloride (Hoffmann-La Roche Ltd., Basel, Switzerland), and crystal violet (Sigma) were dissolved in water. Pradimicin (Bristol-Myers Squibb Company, New Brunswick, N.J.) was dissolved in water at pH 6.8 with 0.2 N NaOH. Each plate contained 15 ml of agar. The drugs were diluted in the corresponding solvents to achieve the concentrations used in the YEPD plates. Preliminary tests were performed to optimize the drug concentrations in the YEPD plates so that growth differences between the different *C. albicans* strains used in the study could be observed. To perform the susceptibility tests, the *C. albicans* strains were grown overnight at 30°C with constant shaking in YEPD liquid medium. The cultures were diluted to 2×10^7 cells per ml in 0.9% NaCl. Five microliters of this suspension and 5 μl of serial dilutions of each yeast culture were spotted onto each type of plate, and the plates were incubated for 2 days at 30°C.

RESULTS

Disruption of the *CDR1* and *BEN*^{r} genes in *C. albicans*. We reported recently the independent isolation of the *C. albicans CDR1* and *BEN*^{r} genes in our laboratory by complementation of hypersusceptibility to fluconazole in *S. cerevisiae* ABC transporter Δ*pdr5* mutant YKKB-13 (2, 27). The *C. albicans CDR1* and *BEN*^{r} genes were first cloned by Prasad et al. (25) by complementation of cycloheximide hypersusceptibility in a similar *S. cerevisiae* mutant and by Fling et al. (8) for conferring resistance to benomyl in *S. cerevisiae*, respectively. Since the *CDR1* and *BEN*^{r} genes were available in our laboratory, the disruption of these genes in *C. albicans* was undertaken with the goal of establishing a correlation between these genes and their involvement in resistance to azole antifungal agents in *C. albicans*. Although the *BEN*^{r} gene was recently disrupted (12), the deletion of this gene was repeated in *C. albicans* CAF4-2, because we wanted to introduce this deletion in a strain with a background lacking a functional *CDR1* gene. Therefore, we constructed plasmids in which substantial parts of these genes were replaced by the *URA3* genetic marker flanked by *hisG* repeats. Linear fragments containing these gene deletion constructs were first generated by excision with specific restriction enzymes (see Material and Methods) and were used to transform strain CAF4-2. Transformations of *C. albicans* were performed in the present study by a lithium acetate transformation method developed in our laboratory. This rapid and convenient method routinely yielded approximately 50 to 100 Ura$^+$ transformants per μg of linearized DNA.

Replacements of the wild-type *CDR1* and *BEN*^{r} alleles with linear fragments were monitored by PCR. The PCR primers (see Fig. 2 and 3) were designed so that at least one of them would anchor outside the genomic region where homologous recombination should occur, thus enabling the analysis of gene

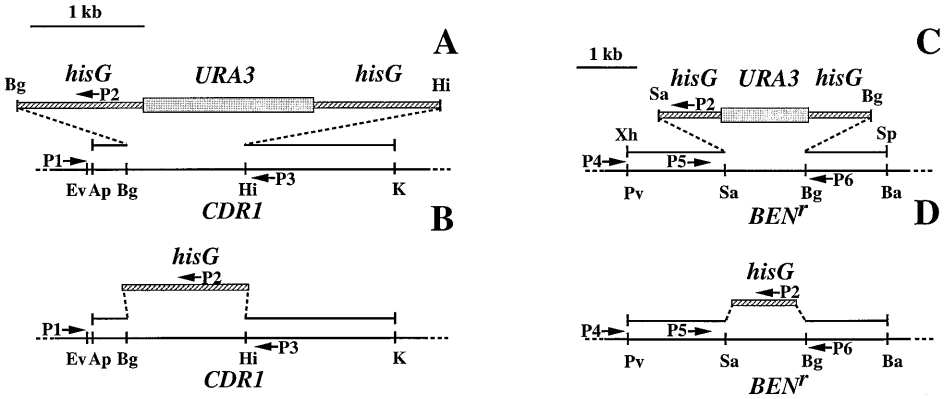

FIG. 1. Restriction maps of the *CDR1* and *BEN*^{r} alleles in disruption experiments. Schematic representations of the disruption of the *CDR1* (A and B) and *BEN*^{r} (C and D) genes before (A and C) and after (B and D) regeneration of the *ura3* genetic marker by selection with 5-FOA. The maps of the wild-type alleles are shown with the corresponding maps of the disrupted alleles. The maps of the disrupted alleles shown in panels A and C match the maps of the linear fragments used for the disruption experiments. The locations of primers P1 to P6 used in PCR to monitor the disruptions of each gene (see Fig. 2 and 3) are indicated. Note that the scale is different between panels A and B and panels C and D. Symbols: Ap, *Apa*I; Ba, *Bam*HI; Bg, *Bgl*II; Ev, *Eco*RV; Hi, *Hin*dIII; K, *Kpn*I; Pv, *Pvu*II; Sa, *Sal*I; Sp, *Spe*I; Xh, *Xho*I.

replacements at the correct genomic loci. Each transformant was subjected to PCR analysis, which was performed with pairs of primers corresponding to the 5′ and 3′ ends of the *CDR1* and *BEN*^{r} genes and with pairs of primers specific for the 5′ ends of these genes and for the *hisG* gene contained in the *URA3* disruption cassette (Fig. 1). The disruption of the first allele of each gene was observed in all the Ura$^+$ transformants tested. Although in each case five Ura$^+$ transformants were analyzed, only the results of PCR with genomic DNAs from two such strains, strains DSY446 and DSY463, are presented here. As expected, PCR performed with primers P1 (specific for the *CDR1* gene) or P4 (specific for the *BEN*^{r} gene) and P2 (specific for the *hisG* gene) with the genomic DNAs of these strains yielded PCR products of 0.8 and 2.8 kb, respectively (Fig. 2 and 3). 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. Only two and three strains out of 5-FOA-resistant strains in the disruption of the first *CDR1* and *BEN*^{r} alleles, respectively, had the correct genotype. Only the results of PCR analysis for one representative strain with each allele regeneration, DSY447 and DSY464, are presented in Fig. 2 and 3. PCR products from strain DSY447 were obtained by using primers P1 and P3; their sizes were 1.55 and 1.4 kb, corresponding to the sizes expected from the Δ*cdr1*::*hisG* disrupted allele and the wild-type allele, respectively (Fig. 2). PCR products were detected from DSY464 by using primers P5 and P6; their sizes were 1.7 and 1.4 kb and corresponded to the sizes of the Δ*ben*::*hisG* disrupted allele and of the wild-type allele, respectively (Fig. 3). Linear fragments containing gene deletion constructs were then reused for the disruption of the second allele of each gene. PCR analysis was again used to select Ura$^+$ transformants in which both alleles were disrupted (see Fig. 2 for analysis of DSY448 and Fig. 3 for analysis of DSY465). On this selection basis, the Ura$^+$ Δ*cdr1* and Δ*ben* homozygous deletion mutants DSY448 and DSY465 could be successfully isolated. Only 2 of 14 Ura$^+$ transformants in the case of the *CDR1* disruption and 1 of 8 Ura$^+$ transformants in the case of the *BEN*^{r} disruption had this correct genotype. The other transformants in both cases were still heterozygotes for the gene deletions, since homologous recombination of the respective disruption cassettes occurred with the first disrupted Δ*cdr1*::*hisG* and Δ*ben*::*hisG* alleles rather than with the wild-type *CDR1* and *BEN*^{r} alleles.

A double Δ*cdr1* Δ*ben* homozygote deletion mutant, DSY468, could be obtained by the same procedures described

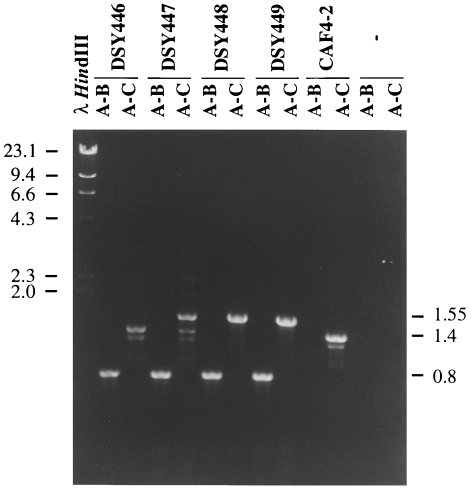

FIG. 2. PCR monitoring of the disruption of the *CDR1* gene. PCR was performed as indicated in Materials and Methods, and the PCR products were electrophoresed in a 1% agarose gel containing ethidium bromide. The sequences of primers P1, P2, and P3 were 5′-AACACCGTTATAGTTGAGACG G-3′, 5′-CGCGCGATACAGACCGGTTC-3′, and 5′-CTAGTGGCATTATCC CAACATTGG-3′, respectively. The expected product size for amplifications with primers P1 and P2 is 0.8 kb on a Δ*cdr1*::*hisG-URA3-hisG* disrupted allele, whereas no amplification should be obtained with the wild-type allele. Amplification from the second *hisG* site with primers P1 and P2, which should yield a 3-kb product, was not observed, probably because of either the limiting length of elongation of *Taq* polymerase or competition with the smaller PCR product of 0.8 kb. The expected product sizes with primers P1 and P3 are 1.4 and 1.55 kb for the wild-type allele and the Δ*cdr1*::*hisG* disrupted allele after 5-FOA treatment, respectively. These expected PCR product sizes correspond to those observed by horizontal gel electrophoresis. The sizes of the molecular mass standards (bacteriophage λ restricted by *Hin*dIII) and the PCR products that were obtained are indicated (in kilobases) to the right and left of the figure. The origin of genomic DNA for each PCR is indicated, along with the corresponding primers (A, P1; B, P2; C, P3).

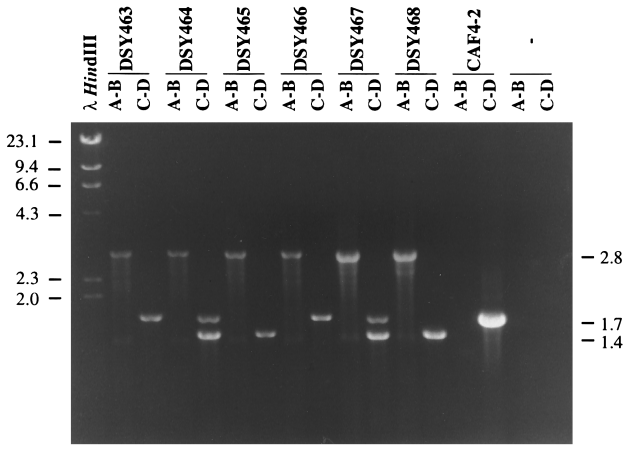

FIG. 3. PCR monitoring of the disruption of the *BEN*^{r} gene in CAF4-1 and in the Δ*cdr1* mutant DSY449. The sequences of the primers P4, P2, P5, and P6 were 5′-TATCGCGTACCTCATCAGCC-3′, 5′-CGCGCGATACAGACCGGTTC-3′, 5′-CAATGACACCTCCTAATTAGC-3′, and 5′-AAAAGCTTCTAATTAGCATACTTAGATCTT-3′, respectively. The expected product size for amplifications with primers P4 and P2 is 2.8 kb on a Δ*ben*::*hisG-URA3-hisG* disrupted allele, whereas no amplification should be obtained with the wild-type allele. The expected product sizes with the primers P5 and P6 are 1.7 and 1.4 kb for the wild-type allele and the Δ*ben*::*hisG* disrupted allele after 5-FOA treatment, respectively. These expected PCR product sizes correspond to those observed by horizontal gel electrophoresis. The origin of genomic DNA for each PCR is indicated, along with the corresponding primers (A, P4; B, P2; C, P5; D, P6). For other details, see the legend to Fig. 2.

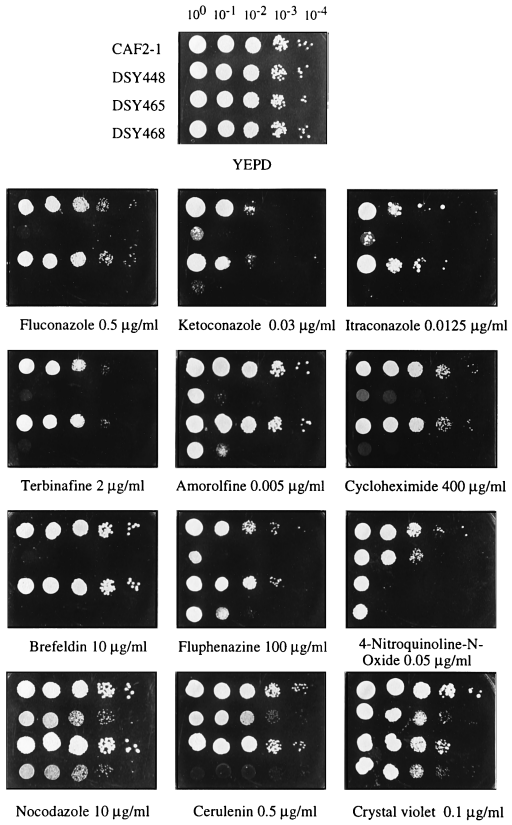

FIG. 4. Susceptibilities of multidrug transporter mutants to antifungal agents and metabolic inhibitors. Each strain was spotted onto agar plates containing complex medium, as indicated in Materials and Methods, and was incubated for 2 days at 30°C. The concentrations of the compounds used in the study are indicated for each plate.

above. The strain background for DSY468 was the Δ*cdr1* mutant DSY449, which originated from DSY448 after 5-FOA treatment. The results of PCR analysis of two intermediate strains (DSY466 and DSY467) in the construction of the double Δ*cdr1* Δ*ben* deletion mutant are presented in Fig. 3, together with the results of analysis of strain DSY468. A correct genotype with homozygous Δ*cdr1* Δ*ben* deletions could be obtained for 2 of 16 Ura$^+$ screened transformants.

Two further verifications of the described disruptions were undertaken. One consisted of Southern blot analysis of deletion mutants DSY448, DSY465, and DSY468. With *hisG* as a probe, signals of the correct length, and no additional signals other than those that were expected, were observed. The results of this analysis therefore corresponded to those of the PCR analysis, which are consistent with the correct genotypes of the deletion mutants. The other verification was performed by Northern blot analysis with total RNA from the same deletion mutants to measure the residual transcription of the *CDR1* and *BEN*^{r} genes. As expected, no *CDR1* mRNA was detected in the Δ*cdr1* or Δ*cdr1* Δ*ben* mutants and no *BEN*^{r} mRNA was detected in the Δ*ben* or Δ*cdr1* Δ*ben* mutants (data not shown).

At this point it is important to mention that the deletion of both the *CDR1* and *BEN*^{r} genes did not affect the viabilities of the mutants that were obtained. The growth of the mutants could not be distinguished from that of *C. albicans* CAF2-1, as judged by the colony sizes of the different yeast strains in Fig. 4 (panel labeled YEPD) and by growth rate measurements (data not shown). The Ura$^+$ Δ*cdr1*, Δ*ben*, and Δ*cdr1* Δ*ben* mutants created in the present study, namely, DSY448, DSY465, and DSY468, respectively, were used subsequently in drug susceptibility assays.

Susceptibilities of multidrug transporter mutants to azole antifungal agents and other inhibitors. It is known that multidrug transporters in the yeast *S. cerevisiae* can accept structurally unrelated compounds as substrates (1), but it is not yet clear to what extent this characteristic applies to different classes of known antifungal agents and metabolic inhibitors in *C. albicans*. Therefore, a panel of different compounds representing these chemicals was chosen to test the susceptibilities of the constructed mutants. Many of these compounds inhibit the growth of *C. albicans*; therefore, it is possible to assign them to putative substrates of multidrug transporters by visualization of growth variations between different yeast types on agar plates containing the different inhibitors. The reduced growth of a given mutant in the presence of a defined inhibitor can be the consequence of an increased accumulation of the substance because of the absence of its specific efflux transporter.

Figure 4 presents the results of this assay for the compounds rendering the multidrug transporter mutants more susceptible than the control wild-type *C. albicans* strain. The Δ*cdr1* mutant DSY448 was rendered hypersusceptible to the azole antifungal agents fluconazole, ketoconazole, and itraconazole, whereas the growth of the Δ*ben* mutant DSY465, surprisingly, was not significantly altered under these conditions (Fig. 4).

The intracellular concentration of fluconazole was measured as described previously (27) with ^{3}H-labelled fluconazole in the same mutants to establish a correlation with the drug susceptibility testing. The intracellular levels of fluconazole in *C. albicans* CAF2-1 (wild type), DSY448 (Δ*cdr1*), DSY465 (Δ*ben*), and DSY468 (Δ*cdr1* Δ*ben*) were 670 ± 30, 1,780 ± 40,

713 ± 50, and 1,677 ± 35 cpm/10^7 cells, respectively (values are the means of two separate experiments; 10^3 cpm is equivalent to 0.2 pmol of fluconazole). These results indicate that cells of the Δ*cdr1* mutant accumulate 2.7 times more fluconazole than wild-type cells. This could be attributed to a less efficient fluconazole efflux activity because of the absence of the ABC transporter Cdr1 in the Δ*cdr1* mutant. Intracellular levels of [^{3}H]fluconazole in the Δ*ben* and the double Δ*cdr1* Δ*ben* mutant do not significantly differ from those in the wild-type strain and the single Δ*cdr1* mutant, respectively. These data demonstrate the role of the multidrug transporter Cdr1 in the efflux of fluconazole in *C. albicans*.

Other medically relevant antifungal agents were tested with the constructed mutants. Among them, terbinafine and amorolfine, which are compounds inhibiting squalene epoxidase and sterol Δ^{14}-reductase or Δ^8-Δ^7 isomerase, respectively (28), significantly reduced the growth of DSY448 compared with that in the wild type, strain CAF2-1 (Fig. 4). Other metabolic inhibitors with different cellular targets such as cycloheximide (a protein biosynthesis inhibitor) (21), brefeldin A (an inhibitor of organelle assembly) (14), and fluphenazine (a caldomulin antagonist) (15) had effects similar to those of the azole derivatives on DSY448. NQO (a mutagen) (4), nocodazole (an inhibitor of tubulin assembly) (31), cerulenin (an inhibitor of fatty acid biosynthesis) (20), and crystal violet (an antifungal agent with an unknown target) (6) were less potent than the other compounds mentioned above at inhibiting the growth of DSY448 compared with the growth of the wild-type strain, CAF2-1 (Fig. 4).

The Δ*ben* mutant DSY465 was not affected by these compounds except when it was grown in the presence of 4-NQO (Fig. 4), which is in accordance with the observations made by Goldway et al. (12), who constructed a Δ*ben* deletion mutant in a *C. albicans* strain with a slightly different genetic background. Those same investigators reported that their single Δ*ben* deletion mutant was rendered slightly more susceptible to cycloheximide than the wild-type parent. This was not observed in our study (Fig. 4), and this might reflect variations in sensitivity of the methods used in both studies to measure drug susceptibility. The effect of the *BEN*^{r} gene deletion could, however, best be observed in a Δ*cdr1* background because it is present in DSY468, a strain which could not be constructed by Goldway et al. (12), since at that time the *CDR1* gene was not available and no prediction on the involvement of multidrug transporters in azole resistance in *C. albicans* had been made. As observed in Fig. 4, the double Δ*cdr1* Δ*ben* mutant DSY468 showed increased growth inhibition in plates containing cycloheximide and cerulenin compared with the growth of strain CAF2-1 and of the Δ*ben* mutant DSY465. A slight increase in the level of inhibition of DSY468 compared with that of the Δ*cdr1* mutant DSY448 was observed with cycloheximide, whereas this effect was more severe with cerulenin.

A number of other compounds failed to inhibit the growth of the mutants constructed in the present study, even if they were used at high concentrations in YEPD agar plates. These substances were amphotericin B (a membrane-destabilizing compound) (11), flucytosine (a toxic pyrimidine analog) (18), nikkomycin Z (a chitin synthase inhibitor) (30), pradimicin (which binds to mannan in the presence of Ca^{2+}) (10), griseofulvin (an inhibitor of microtubule aggregation) (18), hygromycin B (a protein synthesis inhibitor) (13), or phleomycin (an agent causing scission of DNA) (19).

Taken together, the data presented in Fig. 4 indicate that azole derivatives can indeed be used as substrates for the ABC transporter Cdr1, but that other unrelated substrates, as mentioned above, can also serve as substrates for this efflux ABC transporter.

DISCUSSION

In the present study we used *C. albicans* mutants of multidrug transporters to examine the involvement of these transporters in the efflux of different antifungal agents and metabolic inhibitors. The design of homozygous deletion mutants of a given gene is now greatly facilitated in *C. albicans* by the use of the CAF4-2 strain and the *hisG-URA3-hisG* disruption cassette designed by Fonzi and Irwin (9). We have used in the present study two methods to reduce the time needed to construct such mutants, i.e., a simple transformation method with lithium acetate which has not yet been reported and PCR analysis of the transformants. The transformation protocol allowed us to recover enough transformants for PCR analysis after 2 to 3 days of incubation on selective medium. The PCR method could analyze rapidly each step of the sequential disruptions of the *CDR1* and *BEN*^{r} genes. Both procedures were useful for selecting the correct transformants.

The construction of multidrug transporter mutants made possible the testing of their susceptibilities to different toxic compounds. We reasoned that changes in the susceptibility of a given mutant to these compounds would reflect their assignment as substrates for the different multidrug efflux transporters. Thus, it was possible to establish azole derivatives and other important antifungal agents (terbinafine and amorolfine) and other inhibitors mentioned in Fig. 4 as substrates for Cdr1. However, the susceptibility assay used in the study also has some limitations. (i) First, we observed that although fluconazole is recognized as a substrate for Ben^r (27), when the gene for this protein is overexpressed in *S. cerevisiae*, the susceptibility test described here failed to detect this feature. Accordingly, the intracellular contents of fluconazole were almost identical in the Δ*ben* mutant and in the wild-type cells (see Results). The likely explanations for this behavior are that Cdr1 is still functional in a single Δ*ben* mutant and compensates for the absence of Ben^r or that the *BEN*^{r} gene is expressed at a low level in *C. albicans*, and thus, its effect on fluconazole resistance can only be observed when it is expressed at a high level. In fact, we have already observed that *BEN*^{r} is expressed at much lower levels than, for example, *CDR1* in clinical isolates still susceptible to fluconazole (27) and that *BEN*^{r} is also expressed at low levels in CAF4-2, which is the parent strain used for the construction of the mutants in the present study (data not shown).

(ii) Second, it is not possible to test by this simple method compounds which do not inhibit cell growth, although they may represent substrates for efflux transporters. One such example is rhodamine 123, which is believed to be a substrate of *C. albicans* multidrug transporters (3), although it does not inhibit cell growth.

(iii) Third, the effect of deletion of an individual multidrug transporter may be masked by the presence of additional similar transporters in *C. albicans*. This hypothesis is supported by the fact that multidrug transporters exist as multigene families in yeasts. For example, the *S. cerevisiae* genome contained as many as 33 membrane proteins belonging to the class of major facilitators subdivided into two multidrug resistance families (22) and at least 20 genes with similarity to ABC transporters, some of which are involved in multidrug resistance (5). Such a situation is likely to exist in *C. albicans*. Therefore, it will be necessary in the future to clone additional multidrug transporter genes in *C. albicans* to create mutants with multiple gene deletions. To study the ability of individual multidrug transporters to accept diverse compounds as substrates, one possibility would be to use an in vitro reconstituted assay of drug accumulation in the future. This could be achieved, for

example, by the overexpression of transporter genes in baculovirus or in yeasts, in which vesicles enriched with these gene products can be obtained (7).

Now that more evidence that azole and other antifungal agents are substrates for the efflux ABC transporter Cdr1 has been provided, it would be interesting in the future to test new antifungal agents by the same procedure. Since a number of clinical *C. albicans* isolates resistant to azole antifungal agents overexpress multidrug transporter genes and thus can potentially become resistant to other antifungal agents, it would be beneficial to develop drugs that are not used as substrates by multidrug efflux transporters. Screening for such drugs could be performed by the test described here. Furthermore, one can expect that the function of multidrug transporters can be blocked by specific agents. In such a case, resistance to azole and other antifungal agents caused by failure of accumulation could be reversed. This would perhaps reduce the level of resistance of *C. albicans* to azole and other antifungal agents that is now often described in correlation with the failure of treatment with these agents (26).

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