ANTIMICROBIAL AGENTS AND CHEMOTHERAPY, Nov. 1995, p. 2378–2386 Vol. 39, No. 11
0066-4804/95/$04.00+0

Mechanisms of Resistance to Azole Antifungal Agents in *Candida albicans* Isolates from AIDS Patients Involve Specific Multidrug Transporters

D. SANGLARD,[1]* K. KUCHLER,[2] F. ISCHER,[1] J.-L. PAGANI,[3] M. MONOD,[4] AND J. BILLE[1]

Institut de Microbiologie,[1] Division Maladies Infectieuses,[3] and Service de Dermatologie,[4] Centre Hospitalier Universitaire Vaudois, 1011 Lausanne, Switzerland, and Department of Molecular Genetics, University of Vienna Biocenter, 1013 Vienna, Austria[2]

Received 31 May 1995/Returned for modification 14 July 1995/Accepted 14 August 1995

Azole antifungal agents, and especially fluconazole, have been used widely to treat oropharyngeal candidiasis in patients with AIDS. An increasing number of cases of clinical resistance against fluconazole, often correlating with in vitro resistance, have been reported. To investigate the mechanisms of resistance toward azole antifungal agents at the molecular level in clinical *C. albicans* isolates, we focused on resistance mechanisms related to the cellular target of azoles, i.e., cytochrome $P450_{14DM}$ (14DM) and those regulating the transport or accumulation of fluconazole. The analysis of sequential isogenic *C. albicans* isolates with increasing levels of resistance to fluconazole from five AIDS patients showed that overexpression of the gene encoding 14DM either by gene amplification or by gene deregulation was not the major cause of resistance among these clinical isolates. We found, however, that fluconazole-resistant *C. albicans* isolates failed to accumulate ^{3}H-labelled fluconazole. This phenomenon was reversed in resistant cells by inhibiting the cellular energy supply with azide, suggesting that resistance could be mediated by energy-requiring efflux pumps such as those described as ATP-binding cassette (ABC) multidrug transporters. In fact, some but not all fluconazole-resistant clinical *C. albicans* isolates exhibited up to a 10-fold relative increase in mRNA levels for a recently cloned ABC transporter gene called *CDR1*. In an azole-resistant *C. albicans* isolate not overexpressing *CDR1*, the gene for another efflux pump named *BEN*^{r} was massively overexpressed. This gene was cloned from *C. albicans* for conferring benomyl resistance in *Saccharomyces cerevisiae*. Therefore, at least the overexpression or the deregulation of these two genes potentially mediates resistance to azoles in *C. albicans* clinical isolates from AIDS patients with oropharyngeal candidiasis. Involvement of ABC transporters in azole resistance was further evidenced with *S. cerevisiae* mutants lacking specific multidrug transporters which were rendered hypersusceptible to azole derivatives including fluconazole, itraconazole, and ketoconazole.

AIDS is characterized by a marked depression of cellular immunity, often leading to multiple opportunistic infections and, in particular, fungal infections. Retrospective surveys have revealed that 60 to 80% of human immunodeficiency virus-infected patients develop one or more fungal infections at some time during their illness, the most frequent being oropharyngeal candidiasis (OPC) (8, 26, 30). The major agent of OPC is *Candida albicans*, although other yeast species such as *Candida tropicalis*, *Candida glabrata*, or *Candida krusei* have also been implicated.

Treatment of *C. albicans* infections has been greatly facilitated since the introduction of azole antifungal agents, in particular, fluconazole. Because of its solubility in water and its high degree of bioavailability after oral administration, this azole has been used extensively to treat a wide range of *Candida* infections (39). The level of use of this azole for the treatment of OPC in AIDS patients has also risen dramatically. Usually, the clinical response to fluconazole in patients with OPC is satisfactory, but relapses or reinfections frequently occur, probably because of an incomplete eradication of yeast cells in treated patients. Therefore, many AIDS patients have received fluconazole for long periods of time or even for prophylaxis. These repeated treatment courses have led to multiple exposures of *Candida* species to azoles with a resulting development of resistance to fluconazole (for reviews, see references 29, 32, and 35). Indeed, some reports described a correlation between a clinical outcome and in vitro resistance, in which the fluconazole MICs for nearly isogenic *Candida* isolates increased dramatically during antifungal treatment (4, 24, 34, 36, 37, 45).

There are a limited number of reports on the mechanisms of azole resistance in clinical *C. albicans* isolates. The yeast strains used in those studies were isolated from patients after the failure of treatment with the azole derivatives ketoconazole and, more recently, fluconazole (49). Distinct resistance mechanisms have been proposed. One is a reduced permeability of several *C. albicans*-resistant isolates to azole antifungal agents, possibly involving changes in the phospholipids and the sterol membrane composition (16–18, 20, 38). Another resistance mechanism was documented in a separate azole-resistant clinical *C. albicans* strain by Vanden Bossche et al. (47) in which the target of the antifungals agents, the cytochrome $P450_{14DM}$ (14DM), was still active but had a lower affinity toward azoles. Howell et al. (21) investigated other azole-resistant clinical *C. albicans* strains and observed that they exhibited a high fecosterol content, which indicated a partial inhibition of the sterol $\Delta^{5,6}$-desaturase system. This effect is known to establish not only fluconazole and ketoconazole resistance in *Saccharomyces cerevisiae* but also resistance to the polyene macrolide antifungal agents, including nystatin and amphotericin B (50). Resistance mechanisms have also been investigated in other *Candida* species isolated after treatment with azole antifungal agents. For instance, Vanden Bossche et al. (48) reported that

* Corresponding author. Phone: 0041 21 3144083. Fax: 0041 21 3144060. Electronic mail address: dsanglar@eliot.unil.ch.

in a clinical *C. glabrata* isolate cross-resistant to fluconazole, itraconazole, and ketoconazole, the resistance was due to the cumulative effects of reduced permeability to azole derivatives, higher 14DM cellular contents, and higher levels of squalene epoxidase activity. Hitchcock et al. (19) only observed reduced permeability to fluconazole as the major cause of resistance in a separate clinical *C. glabrata* isolate.

Thus, these previous studies indicated that mechanisms leading to azole resistance could be multiple and of different natures. These mechanisms could be dependent on the type of azole used to select for resistance and on the yeast species isolated. Until now, the description of azole resistance mechanisms has been based on the physiological and biochemical properties of azole-resistant isolates which were compared with those of susceptible parent strains. There are, however, practically no published studies of clinical *Candida* isolates exploring the causes of resistance at the molecular level implicating specific genes or gene products. The only exception is for the azole-resistant clinical *C. glabrata* isolate analyzed by Vanden Bossche et al. (48), in which the amplification of the *14DM* gene is related to elevated cellular levels of 14DM, which results in part in azole resistance (49). In the study described in this report, we addressed the molecular basis of azole resistance using sequential clinical *C. albicans* isolates originating from five AIDS patients with OPC treated at the University Hospital of Lausanne (Centre Hospitalier Universitaire Vaudois). Our results suggest the involvement of specific efflux pumps, some of which belong to the superfamily of ATP-binding cassette (ABC) multidrug transporters with the ability to mediate resistance to azole antifungal agents.

MATERIALS AND METHODS

Strains and media. The yeast strains used in the study originated from the Institute of Microbiology (Centre Hospitalier Universitaire Vaudois). The strains were isolated from AIDS patients with OPC. Species determinations were performed by standard protocols. The yeasts were maintained at −80°C. Yeasts were grown in YEPD complex medium with 2% glucose, 2% Bacto Peptone (Difco), and 1% yeast extract (Difco) or in yeast nitrogen base synthetic medium (YNB; Difco) with 2% glucose.

The following *S. cerevisiae* strains were used for antifungal susceptibility testing: YPH499 (*MAT***a** *ura3-52 lys2-801^{amber} ade2-101^{ochre} trp1-Δ63 his3-Δ200 leu2-Δ1*) and YKKB-13 (isogenic to YPH499 but *MAT*α, Δ*sts1*::*TRP1*) (6). YYMI-3 and YYMI-2 (isogenic to YPH499 but *MAT*α Δ*snq2*::*hisG* and *MAT*α Δ*sts1*::*TRP1* Δ*snq2*::*hisG*, respectively) were kindly provided by Y. Mahé. DSY389 is isogenic to YPH499 but contained the plasmid pAAH5/BEN.

Accumulation of [^{3}H]fluconazole in *Candida* isolates. Cells were grown to the mid-log phase in YEPD medium at 30°C with constant agitation. The cells were harvested by centrifugation at 5,300 × *g* at 4°C during 5 min, washed with fresh YNB medium, and resuspended in YNB medium to a cell density of 2.5×10^8 cells per ml. A total of 5 μl of [^{3}H]fluconazole (Amersham) dissolved in ethanol, corresponding to 2.165 nmol with a specific activity of 5×10^6 dpm/nmole, was added to 1 ml of the yeast suspension. The experiment was started by incubating the cells at 30°C in 15-ml Falcon polypropylene tubes (Becton Dickinson, Lincoln Park, N.J.). Samples of 100 μl were withdrawn at different time intervals and were mixed with 0.5 ml of cold YNB medium containing 20 μM unlabelled fluconazole placed in a Spin-X microfiltration unit (pore size, 0.45 μm) with a nylon membrane (Costar, Cambridge, Mass.) adapted to a microcentrifuge tube. The aqueous medium was separated from the cells by a short spin at 9,000 rpm for 30 s at 4°C. The cells were washed with the unlabelled fluconazole-YNB medium three times as described above. The cells were finally resuspended in liquid scintillation medium (Ultima Gold; Packard, Groningen, The Netherlands) and counted in a Beckman counter.

Stock preparations of antifungal agents. The stock solutions of the antifungal agents were made to obtain defined end concentrations of fluconazole (128 to 0.125 μg/ml), ketoconazole (16 to 0.0125 μg/ml), and itraconazole (2 to 0.00195 μg/ml). Fluconazole was dissolved at a concentration of 2.56 mg/ml in water with 7% dimethyl sulfoxide (DMSO), while ketoconazole and itraconazole were dissolved in DMSO at 6.4 and 1.6 mg/ml, respectively. Before addition to the microtiter plate for MIC determinations (see below), ketoconazole and itraconazole were diluted from their stock solutions to 0.32 mg/ml in 20% DMSO and 0.04 mg/ml in 17.5% DMSO, respectively. Fluconazole was a generous gift of Roerig-Pfizer Inc. (New York, N.Y.). Ketoconazole and itraconazole were provided by Janssen Pharmaceutica (Beerse, Belgium).

Determination of MICs. MICs were determined by at least two methods. One protocol (macrodilution method) followed the recommendations of document M27-P of the National Committee for Clinical Laboratory Standards (27). The other method (microdilution method) was adapted from other studies and gave results similar to those obtained by the macrodilution method. In the microdilution method, yeast cells were first grown overnight in YEPD medium, and an inoculum was prepared to a cell density of 1.5×10^4 cells per ml in 0.9% NaCl–0.2% DMSO. Flat-bottom 96-well microtiter plates were prepared by making serial dilutions of the antifungal agents. A broth solution (20 μl; 1× YNB buffered at pH 7 with 1.65 M morpholinepropanesulfonic acid [MOPS] and 10% glucose) was pipetted into each well. To the first well, 20 μl of an appropriate antifungal solution was added, and twofold serial dilutions were performed with a multichannel pipettor, each time transferring 20 μl from one well to another. The last well free of antifungal agent served as a growth control. After completion of the serial dilution, 180 μl of the inoculum was added to each well, making a total of 200 μl per well. Inoculum-free wells were also included. The plates were finally sealed with Parafilm and were incubated at 30°C for 24 and 48 h. For the permanent record, the plates were scanned with a Dynatech MR500 microplate reader (Dynatech Laboratories, Chantilly, Va.) by using a filter at 540 nm. Endpoint readings were set as the antifungal concentration yielding at least 90% growth inhibition after 24 h of growth compared with the growth of the control and were checked also by a visual reading. At the 48-h reading, the MIC-2 score, defined as the lowest drug concentration at which a prominent decrease in cell density could be observed (31), was used. These reading criteria yielded comparable MICs. The *C. albicans* ATCC 90028 strain was used as a quality control for the tests described above.

PCR amplification of *C. albicans* genes and plasmid constructions. The *C. albicans CDR1*, *BEN*^{r}, and *RED* genes encoding the ABC transporter Cdr1, the multidrug transporter Ben^r, and the NADPH cytochrome P450 reductase Red, respectively, were cloned by PCR with 0.2 μg of genomic DNA from the wild-type strain SC5314. PCR buffers and *AmpliTaq* polymerase were from Perkin-Elmer (Roche). The buffer composition was 10 mM Tris-HCl (pH 8.3), 50 mM KCl with 1.5 mM $MgCl_2$ containing 0.2 mM deoxynucleoside triphosphates, and 2.5 U of polymerase per reaction. Briefly, PCR was carried out in a Thermal Cycler 480 (Perkin-Elmer) with a first cycle of denaturation for 4 min at 94°C; this was 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 amplification were as follows: *RED*, 5′-CAA AC(T/A) GGT AC(A/T) GC(A/T) GAA GAT TAT GC-3′ (CPR5) and 5′-(T/C)TA CCA (G/A)AC ATC TTC TTG GTA TC-3′ (CPR3); for *CDR1* (33), 5′-GTT GTT TTG GGG AGA CCC GGT GCT-3′ (CDR1N) and 5′-CTT ACC AGC ACC AGA TGC TCC CAT-3′ (CDR1C); for *BEN*^{r} (10), 5′-AAA AGC TTA TGC ATT ACA GAT TTT TAA GAG-3′ (BENRN) and 5′-AAA AGC TTC TAA TTA GCA TAC TTA GAT CTT-3′ (BENRC).

Since the *C. albicans RED* gene has not yet been isolated, degenerate primers were designed to amplify a conserved region, as deduced from homologous genes in related yeast species. The *C. albicans RED*-amplified fragments were subcloned after blunt ending in the vector pBluescript SK$^+$ (Stratagene, La Jolla, Calif.) digested with *Sma*I. Partial sequencing of the amplified sequence putatively encoding *C. albicans* Red revealed a high degree of homology to the *RED* sequences of other yeasts including *S. cerevisiae*, *C. tropicalis*, and *Candida maltosa*, which are deposited in the EMBL Data Library under the numbers D13788, M35199, and X76226, respectively.

Primers for the amplification of *CDR1* contained the conserved Walker A and Walker B ATP-binding motifs of ABC transporters (33). *CDR1*-amplified fragments with the expected lengths were subcloned into the vector pCRII (Invitrogen Corporation, San Diego, Calif.) and were used subsequently for Northern blot hybridization. Primers for the *BEN*^{r} amplification delimited the entire open reading frame and included *Hin*dIII restriction sites. After *Hin*dIII digestion, *BEN*^{r}-amplified products were subcloned into the same site of the yeast expression vector pAAH5 (1), which allows heterologous gene expression in *S. cerevisiae* by the alcohol dehydrogenase I (*ADHI*) promoter. The resulting plasmid was named pAAH5/BEN and was used to transform *S. cerevisiae* YPH499 by standard protocols (11, 12). *Escherichia coli* DH5α (14) served as a host for the propagation of plasmids. Sequencing of *CDR1*- and *BEN*^{r}-amplified products gave nucleotide sequences identical to those deposited in the EMBL Data Library under accession numbers X77589 (*CDR1*) and X53823 (*BEN*^{r}).

The *URA3-14DM* hybrid probe used for Southern blotting was constructed in two steps. First, *14DM* was amplified by PCR from *C. albicans* SC5314 as described by Burgener-Kairuz et al. (7), blunt-ended, and subcloned at the *Sma*I site of pBluescript SK$^+$, giving pDM. Second, the 1.2-kb *URA3 Eco*RI-*Pst*I fragment from pMK22 (23) was fused to *Eco*RI-*Pst*I-digested pDM, giving pDM/URA3.

Isolation of total yeast RNA and RNA electrophoresis. Yeast cells were grown to the logarithmic growth phase in 100 ml of YNB medium at 30°C with constant shaking. The cells were harvested in 50-ml precooled Falcon tubes (Becton Dickinson) and were centrifuged at 5,300 × *g* for 5 min. The cell pellets were then flash-frozen in liquid nitrogen. The cell pellet was ground to a fine powder under liquid nitrogen in a mortar with a pestle, and the powder was immediately dissolved in a denaturing solution provided by the RNAeasy kit (QIAGEN Inc., Chatsworth, Calif.). The RNA was then isolated according to the recommendations of the supplier. For Northern analysis, RNA of each yeast strain was first

TABLE 1. Characteristics of sequential *C. albicans* strains isolated from five AIDS patients with OPC

Patient no.	*C. albicans* isolates	MIC (μg/ml)[a]			Elapsed time between sampling of isolates (days)	Cumulative dose of fluconazole at the date of isolation (g)	Cumulative dose of other antifungal agents at the date of isolation (g)[b]
		Fluconazole	Ketoconazole	Itraconazole			
I	C23	1.0	0.015	0.0625		4.0	
	C32	8.0	0.0312	0.125	190	6.65	
	C39	32.0	0.125	0.125	194	26.25	2.0 (ITZ) 0.42 (AmB)
II	C45	1.0	0.015	0.0312		1.2	0.6 (ITZ)
	C18	2.0	0.015	0.0625	125	1.4	
	C46	2.0	0.015	0.0625	42	1.4	1.4 (KTZ)
III	C33	0.25	0.015	0.0312			
	C34	2.0	0.015	0.0625	385	0.4	
	C26	>128	4.0	>2.0	494	36.8	0.64 (AmB)
	C82	32.0	0.5	1.0	145	71.2[c]	3.0 (AmB)
IV	C27	1.0	0.015	0.0312		0.4	
	C37	8.0	0.0325	0.0625	605	0.7	
	C40	128.0	2.0	1.0	140	30.5	2.0 (ITZ)
V	C43	0.25	0.015	0.0312			
	C48	64.0	2.0	1.0	202	8.4	
	C56	128.0	4.0	>2.0	61	12.6	2.8 (ITZ)

[a] MICs were determined by the microdilution method as described in Materials and Methods.
[b] Abbreviations: ITZ, itraconazole; KTZ, ketoconazole; AmB, amphotericin B.
[c] Yeast sample was isolated after 3 months of amphotericin B treatment without fluconazole.

denatured in a loading buffer (50% formamide, 100 mM MOPS [pH 7.0], 6.4% formaldehyde, 5% glycerol, 5% of a water solution saturated with bromophenol blue) at 85°C for 5 min and was then subjected to electrophoresis in 1% agarose. The agarose was melted in a buffer containing 0.1 M MOPS, 0.6 M formaldehyde, and 10 μg of ethidium bromide per ml. The electrophoresis buffer was 0.1 M MOPS (pH 7.0). After completion of electrophoresis the RNA was visualized under UV light and the position of the rRNA was determined. Northern transfer was performed overnight on GeneScreen Plus (DuPont NEN, Boston, Mass.) with 10× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate) as a transfer buffer. RNA was fixed on the membrane by baking at 80°C under vacuum.

DNA probe hybridizations and Northern blot quantification. Membranes were prehybridized at 42°C with a buffer consisting of 50% formamide, 1% sodium dodecyl sulfate (SDS), 4× SSC, 10% dextran sulfate, and 100 μg of salmon sperm DNA per ml. ^{32}P-labelled DNA probes were generated by random priming (9) and were added to the hybridization solution overnight. The washing steps were identical to those recommended by the supplier (DuPont NEN). Membranes were then exposed to X-OMAT AR film (Eastman Kodak Company, Rochester, N.Y.) for documentation. For quantitative analysis of the signals, the membranes were exposed for several hours to a phosphor screen, which was subsequently analyzed by a PhosphorImager (Molecular Dynamics, Sunnyvale, Calif.). In Northern blots the *TEF3* mRNAs were analyzed with a 0.7-kb *Eco*RI-*Pst*I fragment from pDC1 as described by Hube et al. (22). *14DM* mRNAs were analyzed with a 1.2-kb *Pst*I-*Bam*HI fragment from pDM. *RED*, *CDR1*, and *BEN*^{r} mRNAs were detected by specific probes generated by PCR as described above.

RESULTS

Characteristics of the *C. albicans* strains isolated from AIDS patients. From the available collection of yeast strains isolated at the Centre Hospitalier Universitaire Vaudois from AIDS patients with OPC, we selected consecutive *C. albicans* isolates (three to four per patient) from five patients. These isolates were chosen on the basis of their increasing fluconazole resistance, as measured by in vitro methods, with the exception of isolates from patient II, which showed no significant increase in fluconazole resistance (Table 1). The isolates from this patient were included in the study as a control for the absence of the resistance mechanisms described later. The MICs of ketoconazole and itraconazole for each isolate were also obtained so that cross-resistance could be evaluated. Table 1 shows that the decrease in susceptibility to fluconazole observed among the isolates from a given patient was also measured for ketoconazole and itraconazole, thus suggesting the azole cross-resistance of these strains. However, the cross-resistance did run in parallel for every isolate. For instance, isolates from patient III, IV, and V were fully cross-resistant to all three azole derivatives, while isolates from patient I increased their level of resistance to fluconazole significantly and to ketoconazole partially (see also Discussion). Table 1 also includes records taken from the patients' charts on antifungal treatments, and particularly on azole therapy. One can observe that the patients who received high doses of fluconazole carried yeast isolates less susceptible to azoles. For these patients, the cumulative doses ranged from 12.6 g (patient V) to 71.2 g (patient III) of fluconazole. The cumulative doses calculated for patient II, whose isolates did not develop fluconazole resistance, did not exceed 1.4 g. This supports the idea that high fluconazole doses are required for the appearance of resistance, as stated by others (24). The susceptibility of the strains listed in Table 1 to the three azole antifungal agents remained unchanged even after subculture in azole-free YEPD over 80 generations (data not shown). This stability suggests that the decrease in azole susceptibility was due to alterations in the yeast genome rather than to adaptations of the cellular metabolism to the presence of azoles. All of the strains listed in Table 1 remained susceptible to amphotericin B. Since amphotericin B resistance in yeasts is coupled with alterations in the ergosterol biosynthetic pathway (50), this suggests that this pathway was still fully operating in these strains.

Successive *C. albicans* strains isolated from a given patient (Table 1) were characterized and compared by different genotyping methods, including the use of the Ca3 repetitive probe (40) and multilocus enzyme electrophoresis (42). These methods showed that the *C. albicans* strains isolated from a given patient were isogenic, whereas strains isolated from different patients significantly differed from each other (6a). This result is in agreement with the idea that the azole-susceptible isolates

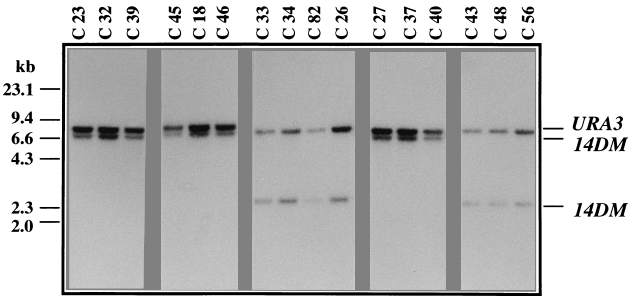


FIG. 1. Relative *14DM* copy number in azole-susceptible and -resistant *C. albicans* isolates from five AIDS patients. Genomic DNA was extracted as described previously (41), and approximately 2 μg of each sample was digested with *Hin*dIII. After electrophoresis, digestions were transferred to a GeneScreen Plus nylon membrane and probed with a *URA3-14DM* hybrid probe to determine a possible relative increase in the *14DM* copy number compared with that of *URA3*. The hybrid probe was constructed as described in Materials and Methods. The corresponding bands for *URA3* and *14DM* are indicated. Although the specific *14DM* signals show restriction site polymorphism, the signal profiles remained identical within samples from a given patient. The *URA3-14DM* hybrid probe was generated from pDM/URA3 (see Materials and Methods) as a 2.4-kb *Eco*RI-*Bam*HI fragment. Details on yeast isolates are described in Table 1.

from a given patient have acquired resistance by specific mutations at some time during antifungal therapy.

Involvement of 14DM in azole resistance. Since the yeast 14DM catalyzing the 14α-demethylation of lanosterol is a specific target of azole antifungal agents in yeasts, resistance to these compounds could be due either to overexpression of 14DM or to changes in the structure of 14DM affecting the affinity to these antifungal agents. Overexpression of 14DM in resistant yeasts could be accomplished theoretically by amplification of the gene for 14DM (*14DM*), up-regulation of transcription, or a higher degree of mRNA stability, all resulting in elevated *14DM* mRNA levels. The gradual increase in resistance observed in sequential isolates from a given patient could well be explained by this phenomenon. However, our results show that the relative copy number of the *14DM* gene compared with that of the *URA3* gene was constant in these strains (Fig. 1), thus eliminating *14DM* amplification as a cause for azole resistance. For the analysis of *14DM* mRNA, total RNA was extracted from the yeast isolates and was subjected to Northern blot analysis (Fig. 2). The *14DM*-specific signals were then quantified after normalization with a *TEF3* probe (Fig. 3). Measurement of *14DM* mRNA levels, as shown in Fig. 3, revealed slight modifications in some of the *C. albicans* isolates. For instance, isolate C46 contained approximately three times more *14DM* mRNA than C45 (Fig. 3B), which is an isolate slightly more susceptible to fluconazole than C46 (Table 1). Isolates C48 and C56, for which MICs of the three azole antifungal agents are high, exhibited a twofold relative increase in *14DM* mRNA levels compared with those in the susceptible parent strain C43 (Fig. 3E). To the contrary, isolate C33, for which azole MICs are low, contained 2.6 times more *14DM* mRNA than C26, for which very high azole MICs have been measured. Taken together, the results presented in Fig. 2 and 3 suggest that the increase in *14DM* mRNA levels in the *C. albicans* isolates was not directly correlated to azole resistance. Moreover, in the azole-resistant isolates, the elevated *14DM* mRNA levels were not prominent and could only partially account for the appearance of resistance. Figure 2 also includes mRNA signals of the NADPH cytochrome P450 reductase gene (*RED*), the product of which constitutes a functional monooxygenase with 14DM. As reported by Truan et al. (46), the Red activity coupled to 14DM can modulate the susceptibility of yeasts toward azole antifungal agents. Those investigators (46) showed that an *S. cerevisiae* mutant lacking Red activity was hypersusceptible to ketoconazole. The data in Fig. 2 and 3 suggest, however, that *RED* mRNA levels were not significantly different in an azole-susceptible *C. albicans* isolate and an azole-resistant one. Thus, the involvement of Red in mediating azole resistance in our isolates seems unlikely.

Accumulation of [^{3}H]fluconazole in azole-susceptible and -resistant strains. Alteration of cell permeability to azoles could be another cause for the appearance of resistance in the *C. albicans* isolates in the present study. This effect has, in fact, recently been documented for a *C. glabrata* clinical isolate resistant to fluconazole (19). Therefore, we incubated cells taken from the mid-log phase with [^{3}H]fluconazole and estimated the intracellular content of this labelled compound in our strains at different time intervals (Fig. 4A to E). The results presented in Fig. 4A to E demonstrate clearly that all azole-resistant strains accumulated less fluconazole than their respective susceptible parents. After 30 min of incubation with [^{3}H]fluconazole, the cells for which the MICs of the three azoles were high (i.e., C26, C39, C40, C48, and C56) reduced their level of fluconazole accumulation by more than 90% compared with those of their susceptible parents. It is also apparent that, among the successive yeast isolates from a given patient, the degree of resistance determined by MICs correlates with a gradual decrease in the level of accumulation of the labelled fluconazole (Fig. 4A, C, and D).

The observed alterations in the permeability of the yeast cells to fluconazole could be due to either a reduced level of drug uptake or an increased level of drug efflux. They could also be the result of changes in the composition of the yeast cell wall and cell membrane. In some prokaryotes and eukaryotes, including mammalian and yeast cells, drug transport can be mediated by specific efflux pumps such as ABC transporters or membrane proteins belonging to the major facilitator superfamily (2, 28). ABC transporters require a source of energy for active transport, and major facilitator superfamily transporters are believed to be dependent on electrochemical potentials. It is possible that such efflux pumps are involved in azole resistance in the clinical isolates examined in the present study. Since specific agents and energy uncouplers interfere with such pumps in higher eukaryotes (13), changes in the level of azole accumulation may be easily observed in yeast cells.

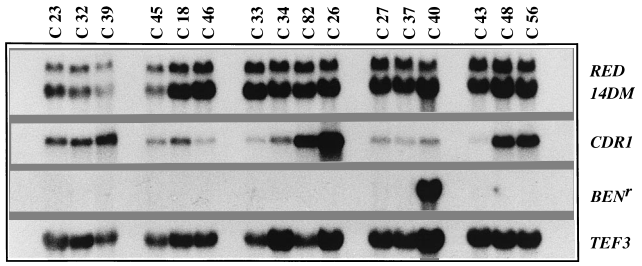


FIG. 2. Northern blot of total RNA from azole-susceptible and -resistant *C. albicans* isolates. Total RNA was extracted as described in Materials and Methods, and 5 μg was loaded for electrophoresis. After transfer to a GeneScreen Plus nylon membrane, the blot was probed sequentially with different labelled DNA fragments (*RED*, *14DM*, *CDR1*, *BEN^r*, and *TEF3*) as indicated on the right. The probes used were those indicated on the right. The origin of each RNA sample is indicated for each isolate from a given patient in the order of the date of isolation. The RNA samples from isolates C26 and C82 were loaded in order of the MICs for the isolates. Sequential hybridizations were performed after stripping each probe in a boiling solution containing 10 mM Tris-HCl (pH 7.0), 1 mM EDTA, and 0.1% SDS. After individual hybridizations and washing steps, membranes were subjected to autoradiography for several hours at −70°C.

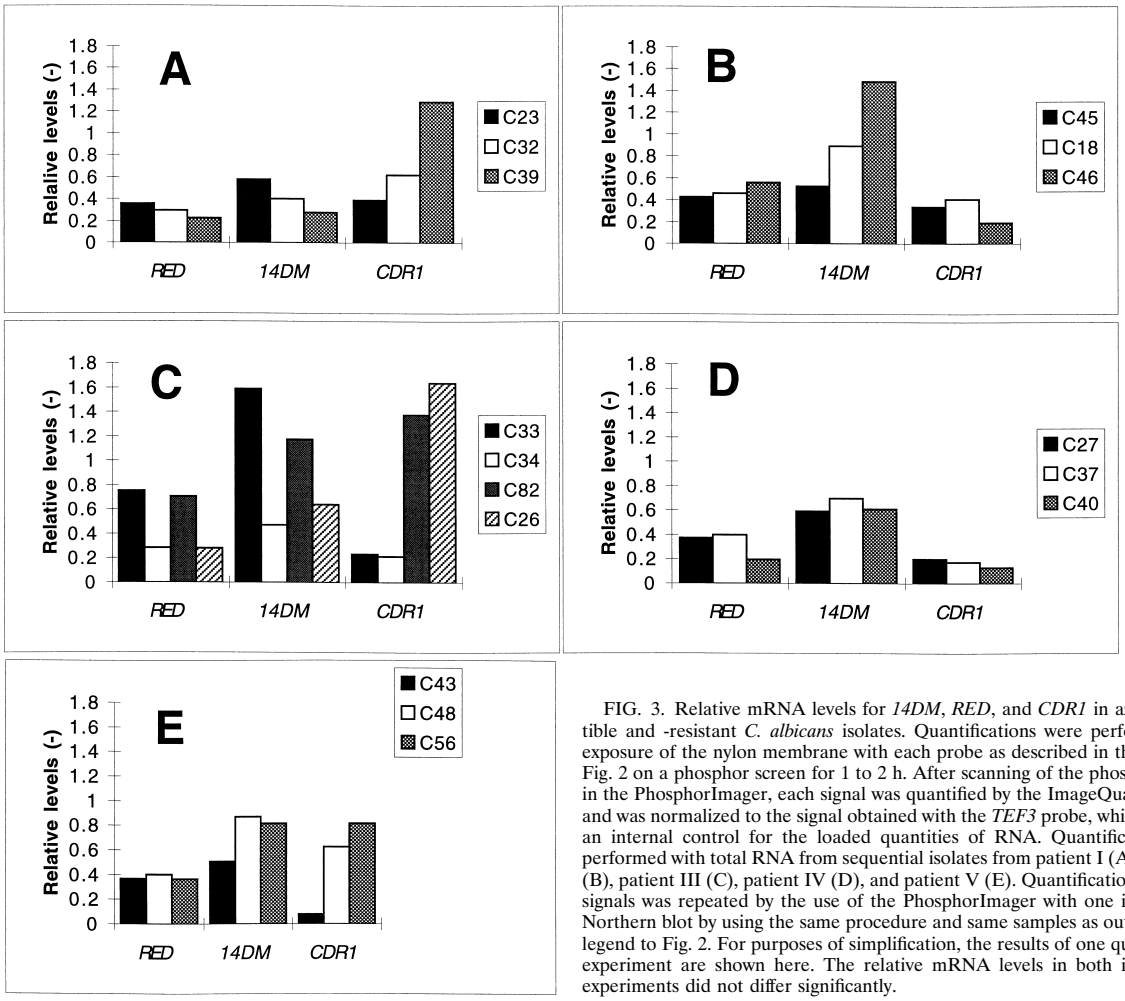
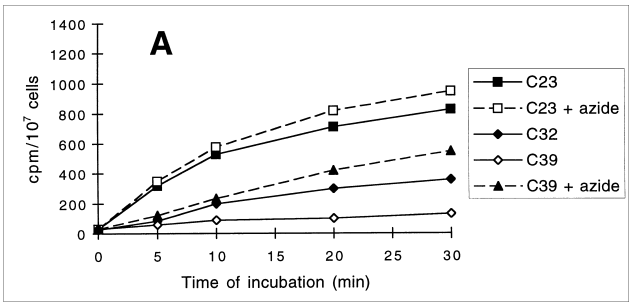
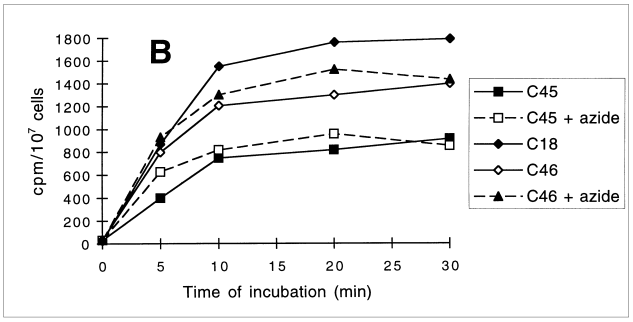


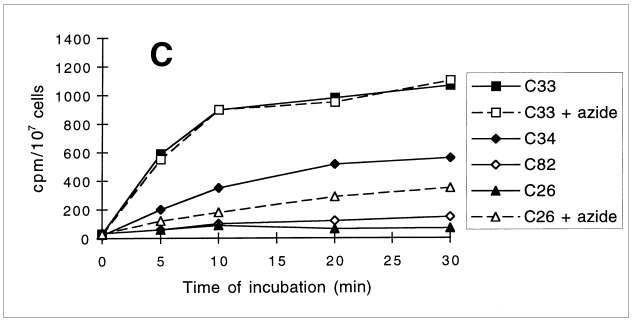
FIG. 3. Relative mRNA levels for *14DM*, *RED*, and *CDR1* in azole-susceptible and -resistant *C. albicans* isolates. Quantifications were performed after exposure of the nylon membrane with each probe as described in the legend to Fig. 2 on a phosphor screen for 1 to 2 h. After scanning of the phosphor screen in the PhosphorImager, each signal was quantified by the ImageQuant software and was normalized to the signal obtained with the *TEF3* probe, which served as an internal control for the loaded quantities of RNA. Quantifications were performed with total RNA from sequential isolates from patient I (A), patient II (B), patient III (C), patient IV (D), and patient V (E). Quantification of mRNA signals was repeated by the use of the PhosphorImager with one independent Northern blot by using the same procedure and same samples as outlined in the legend to Fig. 2. For purposes of simplification, the results of one quantification experiment are shown here. The relative mRNA levels in both independent experiments did not differ significantly.

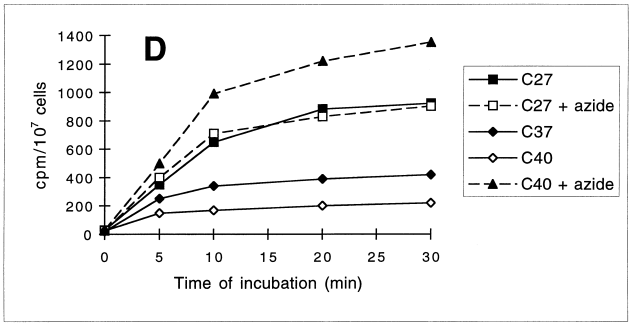
Therefore, fluconazole accumulation was measured in the presence of the reversing agent verapamil or with the energy uncoupler carbonyl cyanide *m*-chlorophenylhydrazone (CCCP) both in azole-susceptible and in azole-resistant yeast cells. However, none of these compounds used at different concentrations (up to 200 μg/ml for verapamil and 50 μg/ml for CCCP) affected the accumulation of fluconazole, probably because of their inability to penetrate the yeast cell wall (data not shown). Alternatively, in order to interfere with the generation of energy in the yeast cell, azide (NaN_3) was used at a subinhibitory concentration in separate experiments. NaN_3 is an agent that affects the generation of ATP in the mitochondria of yeast cells (25). As shown in Fig. 4A to E, incubation of yeast cells in the presence of 0.1 mM NaN_3 generally resulted in an increased level of accumulation of labelled fluconazole. More precisely, the azole-resistant strains C39, C26, C40, and C56 incubated for 30 min in the presence of NaN_3 accumulated 4.2, 5.0, 6.1, and 4.0 times more [^{3}H]fluconazole, respectively, than they did in its absence. In contrast, the susceptible parents of each of these strains did not exhibit such differences in levels of accumulation in the presence of NaN_3. These results suggested that the degree of accumulation of fluconazole is energy dependent in the resistant strain and, thus, possibly involves drug efflux transporters. Moreover, the effect of NaN_3 on the accumulation of [^{3}H]fluconazole in azole-susceptible isolates was not consistent with the presence in yeast cells of an active uptake system for azole antifungal agents. This idea was supported by marginal differences in the levels of [^{3}H]fluconazole accumulation in the presence or absence of NaN_3 observed in the azole-susceptible isolates C23, C45, C33, C27, and C43 (Fig. 4). Taking an active uptake system into consideration, one would have expected a reduced level of accumulation of fluconazole in the presence of NaN_3.

Quantification of mRNA for multidrug resistance genes in *C. albicans* isolates. The results mentioned above prompted us to probe Northern blots of total *C. albicans* RNA with known *C. albicans* genes conferring multidrug resistance. Two of these genes have been cloned at present, i.e., *CDR1* (for *Candida* drug resistance) (33) and *BEN*^{r} (for conferring benomyl resistance in *S. cerevisiae*) (10). *CDR1* belongs to the class of ABC transporters, whereas *BEN*^{r} belongs to the superfamily of major facilitators (2). Figure 2 shows that the levels of *CDR1* mRNA signals were apparently increasing with an increasing degree of azole resistance among the sequential isolates from some patients. Quantification of these signals by phosphor imaging after normalization with a *TEF3* probe (44) indicated that the relative increases in the *CDR1* mRNA level were threefold for C23 to C39 (Fig. 3A), 8-fold for C33 to C26 (Fig. 3C), and 10-fold for C43 to C56 (Fig. 3E). Interestingly, the increase in the signal was related to the MICs of the antifungal









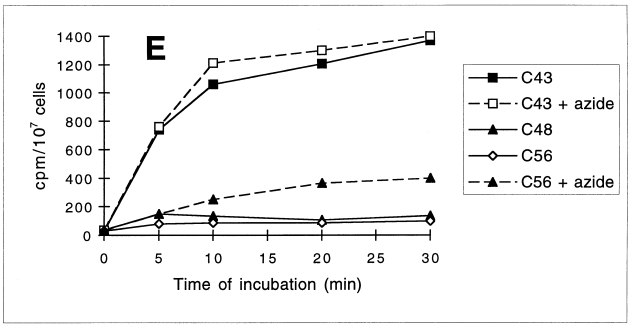


FIG. 4. [^{3}H]fluconazole accumulation in azole-susceptible and -resistant *C. albicans* isolates. Each experiment was carried out twice, and the mean values at each time point are given. The values at each time point varied by less than 15%. Accumulation experiments were performed with sequential isolates from patient I (A), patient II (B), patient III (C), patient IV (D) and patient V (E). When indicated, the azide concentration in the assays was 0.1 mM, which is a value below the MIC of NaN_3 (0.75 mM). The rate of accumulation of fluconazole in azole-susceptible strains from each patient (i.e., C23, C45, C33, C27, and C43) after 5 min of incubation varied between 2.4 and 5.6 pmol/min/10^9 cells.

agents, since the MIC of fluconazole for C39 was 32 μg/ml, whereas the MICs for C26 and C56 were equal to or greater than 128 μg/ml. The increase in the *CDR1* mRNA level was, however, not observed for the sequential isolates C45 to C46 (Fig. 3B) and C27 to C40 from patient IV (Fig. 3D). Whereas the fluconazole MICs were not significantly different for C45, C18, and C46, the MICs for the consecutive isolates from patient IV increased significantly (Table 1). Isolate C40 accumulated significantly less fluconazole than the susceptible parent C27, and the fact that this phenomenon was reversed by incubation with NaN_3 suggested that another energy-dependent transport mechanism could be involved in azole resistance. This hypothesis was supported when the same Northern blot was probed with the *BEN*^{r} probe. In this case, *BEN*^{r} mRNA was detected only in the RNA extracted from strain C40 (Fig. 2). Thus, the lack of an increase in *CDR1* mRNA levels in this strain was apparently compensated for by the massive overexpression of *BEN*^{r}.

Involvement of multidrug transporters in azole resistance in the *S. cerevisiae* yeast model. Different observations now strongly suggest that multidrug transporters could be important for mediating azole resistance in *C. albicans*. We tentatively tested this hypothesis using the yeast *S. cerevisiae*, from which such transporters have been cloned and the corresponding genes have been disrupted. An increasing number of such multidrug transporters have been reported recently in *S. cerevisiae* (2), among which Sts1 (also known as Pdr5 or Ydr1) (3, 6, 15) and Snq2 (43) have been well characterized for their substrate specificities. The *C. albicans* Cdr1 ABC transporter seems to be the homolog of the *S. cerevisiae* Sts1 rather than Snq2, as judged by amino acid sequence alignments (data not shown). We therefore addressed the involvement of these transporters in azole resistance by incubating the parent strain *S. cerevisiae* YPH499 and respective deletion mutants in increasing concentrations of the three azole antifungal agents used in the study. As shown in Fig. 5, the Δ*sts1* deletion mutant strain YKKB-13 behaved as being hypersusceptible to all three azoles tested. The MICs for the Δ*sts1* mutant were 0.5 μg/ml for fluconazole and 0.0625 μg/ml for ketoconazole and itraconazole, whereas these values for the parent strain YPH499 with a functional *STS1* gene were 16, 2, and 1 μg/ml, respectively. Therefore, Sts1 is likely to be specifically involved in the ability of the organism to export azole derivatives outside of the cell. Interestingly the ABC transporter Snq2 does not seem to be involved in such a mechanism, since the Δ*snq2* deletion mutant YYMI-3 was as susceptible as YPH499 to azoles, suggesting that the azole derivatives used here were not substrates for Snq2. It is noteworthy that the double Δ*snq2* Δ*sts1* mutant YYMI-2 was slightly more susceptible to azoles than the single Δ*sts1* mutant, thus revealing a synergism between the two mutations in terms of conferring drug susceptibility. In a separate experiment (Fig. 6), the expression of the *C. albicans CDR1* in the *S. cerevisiae* Δ*sts1* mutant demonstrated directly the involvement of Cdr1 in conferring resistance to azole derivatives. As shown in Fig. 6, this conclusion is supported by the fact that the plasmid pDS243, which was cloned by functional complementation of hypersusceptibility to fluconazole and which con-

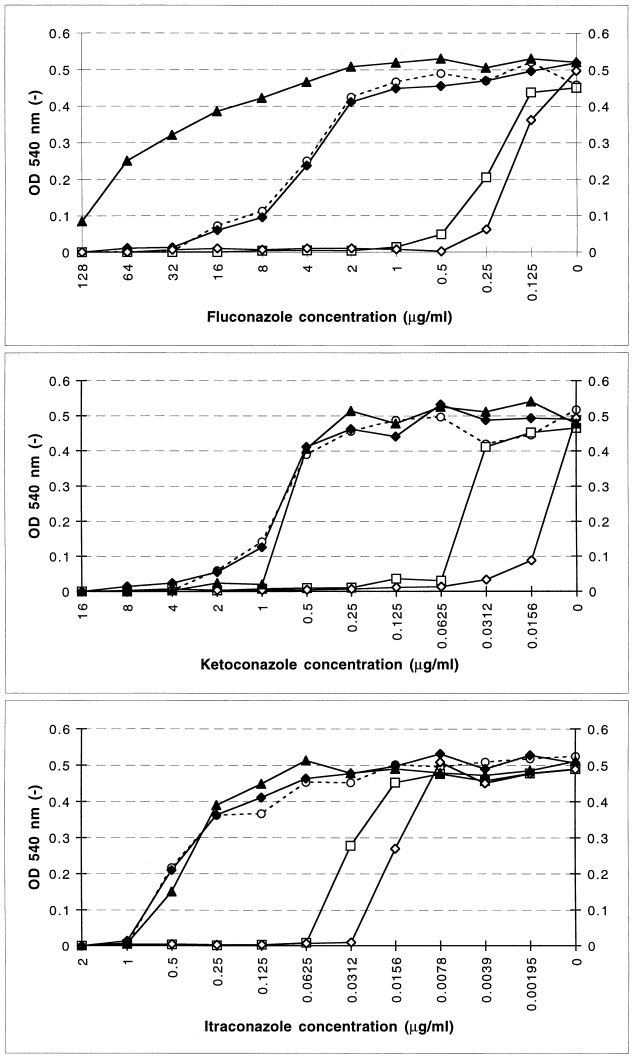


FIG. 5. Susceptibilities of *S. cerevisiae* ABC transporter mutants to azole derivatives. *S. cerevisiae* strains were cultivated overnight in complex YEPD medium and were subjected to the MIC assay by the microdilution method described in Materials and Methods. Assays were performed in duplicate with the wild-type strain YPH499 (○), the Δ*sts1* deletion mutant YKKB-13 (□), the Δ*snq2* deletion mutant YMMI-3 (◆), the double Δ*sts1* Δ*snq2* deletion mutant YMMI-2 (◇), and the transformant DSY389 (▲). The optical density (OD) of each well on the microtiter plate was determined at 540 nm on a microplate reader after 48 h of incubation at 30°C, and each value was plotted against its corresponding antifungal concentration. Duplicate experiments yielded comparable results. The growth medium was supplemented with the required amino acids except for that for isolate DSY389, for which leucine was omitted to maintain the plasmid pAAH5/BEN. YPH499 transformed with the parent strain pAAH5 gave essentially the same growth curve as the untransformed strain, and therefore, only data for YPH499 are included in this analysis.

tained *CDR1*, conferred resistance to all three azole derivatives tested.

The involvement of the *C. albicans* Ben^r multidrug transporter in mediating azole resistance, which was suggested by the results presented in Fig. 2, was tested by overexpressing *BEN*^{r} in *S. cerevisiae* under the control of the *ADHI* promoter. YPH499 was therefore transformed with the plasmid pAAH5/BEN and a transformant (DSY389) assayed in the same test performed as described above (Fig. 5). Figure 5 demonstrates that DSY389 has acquired resistance to fluconazole because of the presence of pAAH5/BEN. The fluconazole MIC for DSY389 was >128 μg/ml, whereas the MIC for YPH499 was still 16 μg/ml, showing that fluconazole is a new substrate for Ben^r. However, overexpression of *BEN*^{r} in DSY389 did not result in the elevation of the MICs of ketoconazole and itraconazole, demonstrating a rather narrow azole substrate specificity for Ben^r. The same observation was made when *BEN*^{r} was cloned directly by functional complementation of hypersusceptibility to fluconazole in YKKB-13 (Fig. 6). As deduced from Fig. 6, the expression of *BEN*^{r} contained in the plasmid pDS245 conferred, as expected from the studies of Benyaacov et al. (5), resistance to benomyl, cycloheximide, and nitroquilonine-*N*-oxide. pDS245, however, conferred resistance only to fluconazole and not to ketoconazole and itraconazole.

DISCUSSION

In the present study we investigated a total of 16 sequential *C. albicans* strains isolated from five AIDS patients. The strains had increasing levels of resistance to fluconazole after prolonged treatment mainly with this antifungal agent. We showed that the *C. albicans* isolates that were less susceptible to fluconazole were not able to accumulate this antifungal agent as efficiently as their susceptible parents collected in the first episodes of OPC. Failure in accumulating this compound among resistant yeast cells can be related to at least two phenomenona: a significant increase in the level of *CDR1* mRNA and a corresponding increase in the level of *BEN*^{r} mRNA. *CDR1* and *BEN*^{r} are both multidrug transporter genes, each belonging to distinct classes of transporters (10, 33). Multidrug resistance mediated by such types of transporters is an effect well documented in microorganisms such as bacteria of medical importance (28) and in different laboratory yeasts including *S. cerevisiae* and *Schizosaccharomyces pombe* (2). It is, however, the first time that multidrug transporters appear to be involved in azole resistance mechanisms in clinical *C. albicans* isolates.

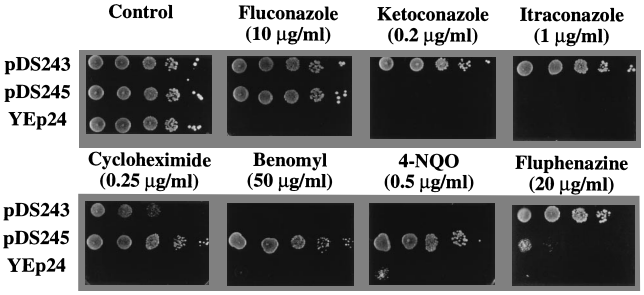


FIG. 6. The *C. albicans CDR1* and *BEN*^{r} genes conferred resistance to specific azole antifungal agents. Plasmids pDS243 and pDS245 were isolated by screening of a *C. albicans* gene library transformed into the *S. cerevisiae* Δ*sts1* mutant YKKB-13 for fluconazole resistance. The gene library was constructed with partially *Sau*3A-digested *C. albicans* SC5314 genomic DNA which was cloned into the *Bam*HI site of YEp24. YKKB-13 was retransformed with pDS243, pDS245, and the parent plasmid YEp24. Each transformant was grown to the mid-log phase at 30°C in minimal medium with the required amino acids, but uracil was omitted. Each culture was diluted to an optical density at 540 nm of 2, and 5 μl of a serial dilution (10^0 to 10^{-4}) was spotted onto YEPD plates containing the desired antifungal agents. The plates were incubated at 30°C for 48 h. pDS243 and pDS245 contained DNA inserts with restriction site patterns identical to those of *CDR1* (33) and *BEN*^{r} (5). Furthermore, PCR products could be obtained with pDS243 and pDS245 as templates by using primers specific for *CDR1* and *BEN*^{r}, respectively. pDS243 conferred resistance to azole derivatives as well as cycloheximide and fluphenazine. pDS245 conferred resistance to cycloheximide, benomyl, and 4-nitroquilonine-*N*-oxide (4-NQO) as described previously (5) and to the azole derivative fluconazole.

Increases in the *CDR1* and *BEN*^{r} mRNA levels in our azole-resistant isolates have not been yet correlated to an increase in the levels of their respective translated products and of their coupled activities in reconstituted in vitro systems. However, there is indirect evidence that the *C. albicans* Cdr1 and Ben^r multidrug transporters can use azoles as substrates. First, the *S. cerevisiae* Δ*sts1* mutant was hypersusceptible to all three azole derivatives used in the study (Fig. 5), which is a strong indication that Sts1, a close homolog of Cdr1, is implicated in their transport. Second, we cloned independently the *C. albicans CDR1* and *BEN*^{r} genes by functional complementation of fluconazole hypersusceptibility of an *S. cerevisiae* Δ*sts1* mutant (Fig. 6). Contrary to *BEN*^{r}, *CDR1* could also complement ketoconazole and itraconazole hypersusceptibility. Moreover, *BEN*^{r} overexpression in *S. cerevisiae* significantly decreased the susceptibility of this yeast to fluconazole (Fig. 5), which confirms that Ben^r is involved specifically in a fluconazole resistance mechanism. Ben^r, which accepts as a substrate not only benomyl but also cycloheximide and 4-nitroquilonine-*N*-oxide (5), therefore does not seem to be involved in the export of ketoconazole and itraconazole. Curiously, isolate C40, which overexpressed *BEN*^{r}, is less susceptible to all three azole derivatives than the parent strain. Since Ben^r can only account for fluconazole resistance, there must be another yet uncharacterized resistance determinant present in this strain. One remaining possibility is that the *BEN*^{r} gene which was expressed in *S. cerevisiae* and which was amplified from SC5314 genomic DNA represents a different allele of *BEN*^{r} than that present in C40 with respect to its substrate specificity. Additional experiments will be required to test this hypothesis.

The increase in the mRNA levels of both the *CDR1* and *BEN*^{r} genes in isolates less susceptible to azoles could be the consequence of different molecular mechanisms. They include higher levels of mRNA stability, gene amplification, or deregulation because of a specific point mutation in the promoter region of each gene or the effect of *trans*-acting factors on these promoters. The molecular basis of multidrug resistance has been investigated in more detail in *S. cerevisiae*. The model with this organism should help to provide an understanding of the mechanisms of resistance that exist in clinical *C. albicans* isolates. Some of the *S. cerevisiae* mutants in the multidrug resistance pathway have been associated with pleiotropic drug resistance loci such as *PDR1*, *PDR3*, *PDR7*, and *PDR9. PDR1* and *PDR3* encode homologous transcription regulators which, when altered by specific mutations, modulate the expression of the ABC transporter gene *STS1* and render *S. cerevisiae* resistant to a number of unrelated chemicals (3, 6). Other transcription regulators implicated in multidrug resistance have been described (YAP1, YAP2) and display resistance to specific compounds (for a review, see reference 2). It is possible that similar transcription regulators modulate the expression of the *CDR1* gene in *C. albicans* and, in addition, other yet uncharacterized genes in the clinical *C. albicans* isolates from the present study.

The two mechanisms of resistance mentioned above can be combined with other effects in an additive manner which may account for the progressive increase in azole MICs measured for yeasts collected during antifungal treatment. This possibility can be illustrated in sequential isolates C27 and C37 from patient IV. The fluconazole MICs for these isolates are 1.0 and 8.0 μg/ml, respectively (Table 1), although no increase in *CDR1* or *BEN*^{r} mRNA levels could be observed (Fig. 2). A combination of several resistance mechanisms has already been proposed by Vanden Bossche et al. (48). The increase in *14DM* mRNA levels observed in the present work in some azole-resistant strains may be one of these accompanying effects, if it is followed, however, by corresponding elevated levels of intracellular 14DM. Another hypothetical combined effect could be changes in the affinity of 14DM to azole derivatives, which could affect the susceptibilities of the *C. albicans* strains investigated here, as was also reported by Vanden Bossche et al. (47) for a specific clinical *C. albicans* isolate. We are testing the affinity of each 14DM from susceptible and resistant isolates to azole derivatives to answer this question.

The synergistic effect of different resistance mechanisms which could decrease the susceptibilities of clinical isolates to azole derivatives can be even more complicated by the fact that individual yeast isolates develop a different degree of susceptibility to each of the azoles tested in our study. This is reflected, for example, in the *C. albicans* isolates C39, C48, and C82. The MICs of fluconazole for these yeasts are similar (32 to 64 μg/ml), but each isolate exhibits different susceptibilities to ketoconazole and itraconazole. For instance, while the MIC of both ketoconazole and itraconazole for isolate C39 are 0.125 μg/ml MICs of the same antifungal agents for isolate C48 are 0.5 and 1.0 μg/ml, respectively, and the MICs for isolate C82 are 4.0 and more than 2.0 μg/ml, respectively. This suggests that resistance mechanisms specific for defined azole antifungal agents must exist. One hypothesis explaining these variations mentioned above could be that the substrate specificity and the turnover rate of Cdr1 itself in C48 and C82 are further altered by specific mutations, in addition to the fact that increased levels of cellular *CDR1* expression are responsible for higher rates of drug efflux in the azole-resistant isolates.

It is apparent from this discussion that other azole resistance mechanisms possibly not involving the azole transport phenomenon could be identified. This conclusion is supported by the observations made with the limited number of *C. albicans* isolates examined in the present study and could be substantiated by analyzing other azole-resistant *C. albicans* isolates from other sources. To characterize additional factors that enable clinical *C. albicans* isolates to acquire resistance to specific azoles, one strategy pursued in our laboratory consisted of transforming a *C. albicans* genomic library into an *S. cerevisiae* Δ*sts1* mutant and selecting for a phenotype of resistance to specific azole derivatives. In fact, this strategy has not only recovered independently the *CDR1* and *BEN*^{r} genes, as mentioned above, but has also recovered at least three other unrelated drug resistance genes whose molecular characterization is now under way.

Once the diversity of resistance mechanisms is established, it would be interesting to test their incidence in other azole-resistant *C. albicans* isolates from patients with repeated episodes of OPC. A specific mechanism may be related to defined OPC treatment modalities, and thus, it could be possible to prevent or delay the appearance of azole resistance in *C. albicans* isolates. The identification of new resistance determinants in yeasts may also give the opportunity to define new targets for the design of alternative and effective antifungal agents.

ACKNOWLEDGMENTS

We are indebted to C. Durussel for maintaining the *C. albicans* strain collection, P. Boerlin for sharing results prior to publication and for discussions, and M. P. Glauser for critical review of the manuscript. We thank Y. Mahé (University of Vienna Biocenter) for yeast strains YYMI-3 and YYMI-2 and B. Hube for *C. albicans* SC5314 and the *TEF3* gene.

The research carried out during this study was supported by a grant from the Austrian Science Foundation (P-10123) (to K.K.) and by a grant from the AIDS Priority Program from the Office Fédéral de la Santé Publique (to J.B. and D.S.).

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