

Role of ATP-Binding-Cassette Transporter Genes in High-Frequency Acquisition of Resistance to Azole Antifungals in *Candida glabrata*

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Candida glabrata has been often isolated from AIDS patients with oropharyngeal candidiasis treated with azole antifungal agents, especially fluconazole. We recently showed that the ATP-binding-cassette (ABC) transporter gene *CgCDR1* was upregulated in *C. glabrata* clinical isolates resistant to azole antifungal agents (D. Sanglard, F. Ischer, D. Calabrese, P. A. Majcherczyk, and J. Bille, *Antimicrob. Agents Chemother.* 43:2753–2765, 1999). Deletion of *CgCDR1* in *C. glabrata* rendered the null mutant hypersusceptible to azole derivatives and showed the importance of this gene in mediating azole resistance. We observed that wild-type *C. glabrata* exposed to fluconazole in a medium containing the drug at 50 µg/ml developed resistance to this agent and other azoles at a surprisingly high frequency (2×10^{-4} to 4×10^{-4}). We show here that this high-frequency azole resistance (HFAR) acquired in vitro was due, at least in part, to the upregulation of *CgCDR1*. The *CgCDR1* deletion mutant DSY1041 could still develop HFAR but in a medium containing fluconazole at 5 µg/ml. In the HFAR strain derived from DSY1041, a distinct ABC transporter gene similar to *CgCDR1*, called *CgCDR2*, was upregulated. This gene was slightly expressed in clinical isolates but was upregulated in strains with the HFAR phenotype. Deletion of both *CgCDR1* and *CgCDR2* suppressed the development of HFAR in a medium containing fluconazole at 5 µg/ml, showing that both genes are important mediators of resistance to azole derivatives in *C. glabrata*. We also show here that the HFAR phenomenon was linked to the loss of mitochondria in *C. glabrata*. Mitochondrial loss could be obtained by treatment with ethidium bromide and resulted in acquisition of resistance to azole derivatives without previous exposure to these agents. Azole resistance obtained in vitro by HFAR or by agents stimulating mitochondrial loss was at least linked to the upregulation of both *CgCDR1* and *CgCDR2*.

The number of life-threatening fungal infections observed worldwide has increased significantly (4). This situation is the consequence of the increasing use of immunosuppressive drugs and antibiotics given to cancer or organ transplant patients. The AIDS epidemic, which maintains human immunodeficiency virus-infected patients permanently immunocompromised, has also largely contributed to the higher incidence of fungal infections. The major agents of fungal infections are *Candida* species, and *Candida albicans* is the most frequent species, followed by *C. glabrata* and *C. tropicalis* (22). The need for antifungal agents has correspondingly increased but is restricted to a few agents. Among these, azole antifungal agents have been more extensively utilized, in particular, fluconazole since it is well tolerated.

Reports on resistance to azole antifungal agents were rare until the late 1980s. The first cases of resistance were reported in *C. albicans* after prolonged therapy with miconazole and ketoconazole (12–14). Fluconazole is used in a wide variety of clinical settings, and antifungal resistance to this agent has been more frequently reported (30). Resistance of yeast clinical isolates to azole antifungal agents can be mediated by the following different mechanisms: (i) the cellular content of the azole cellular target (Erg11p) can be elevated, (ii) the affinity of Erg11p to these agents can be decreased, (iii) cells can fail to accumulate these agents, and (iv) the ergosterol biosynthetic pathway can be altered by inactivation of the sterol $\Delta^{5,6}$ -de-

saturase (25, 27, 29, 31). Since resistance to azole antifungal agents can develop as a stepwise process over time, one can expect these mechanisms to combine with each other (27).

C. glabrata is a yeast with intrinsic low susceptibility to azole derivatives and is often recovered from clinical samples originating from AIDS or cancer patients. This yeast is still able to acquire resistance to azole antifungal agents during treatment with these types of antifungals. We recently described the major mechanism of azole resistance in two separate *C. glabrata* isolates from AIDS patients with oropharyngeal candidiasis who were treated with fluconazole (26). The two azole-resistant isolates were accumulating less fluconazole than their azole-susceptible parents. This effect was due to the upregulation of *CgCDR1*, a gene encoding a multidrug transporter of the ATP-binding-cassette (ABC) transporter family. Deletion of *CgCDR1* in an azole-resistant clinical isolate rendered the resulting mutant susceptible to azole derivatives, and it accumulated as much fluconazole as the azole-susceptible parent. We therefore considered *CgCDR1* to be the major ABC transporter gene involved in the resistance of clinical isolates to azole derivatives. During our investigations on mechanisms of azole resistance in *C. glabrata*, we observed that azole-susceptible isolates exposed in vitro to fluconazole were generating azole-resistant clones at a surprisingly high frequency. In this study, we addressed the mechanisms operating in this process. We show here that the high frequency of azole resistance (HFAR) acquired in vitro is linked with mitochondrial loss and *CgCDR1* upregulation. We also identified another ABC transporter gene, *CgCDR2*, which contributes to this in vitro-acquired resistance.

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TABLE 1. Yeast strains used in this study

Strain	Genotype or phenotype	Parent strain	Reference
<i>S. cerevisiae</i>			
YKKB-13	<i>MATa ura3-52 lys2-801^{amber} ade2-101^{ochre} trp1-Δ63 his3-Δ200 leu2-Δ1 Δpdr5::TRP1</i>	YPH499	5
DSY1693	Contains pAAH5	YKKB-13	
DSY1694	Contains pDS647 (<i>CgCDR2</i> expression plasmid)	YKKB-13	
DSY1696	Contains pDS646 (<i>CgCDR1</i> expression plasmid)	YKKB-13	
<i>C. glabrata</i>			
DSY562	<i>C. glabrata</i> clinical isolate, susceptible to azoles		26
DSY565	<i>C. glabrata</i> clinical isolate, resistant to azoles	DSY562	26
DSY1029	<i>ura3</i>	DSY565	26
DSY1041	<i>Δcgcdr1::hisG-URA3-hisG</i>	DSY1029	26
DSY1612	<i>Δcgcdr2::hisG-URA3-hisG</i>	DSY1029	This study
DSY1613	<i>Δcgcdr2::hisG Δcgcdr1::hisG-URA3-hisG</i>	DSY1056	This study
DSY562-HFAR	HFAR strain	DSY562	This study
DSY1041-HFAR	HFAR strain	DSY1041	This study
DSY562-rho	Mitochondrion-deficient mutant isolated by EtBr treatment	DSY562	This study
DSY1041-rho	Mitochondrion-deficient mutant isolated by EtBr treatment	DSY1041	This study
DSY1613-rho	Mitochondrion-deficient mutant isolated by EtBr treatment	DSY1613	This study

MATERIALS AND METHODS

Strains and media. The yeast strains used in this study are listed in Table 1. They were grown at 30°C on complex medium (yeast extract-peptone-dextrose [YEED]) with 2% (wt/vol) glucose, 1% (wt/vol) Bacto Peptone (Difco), and 0.5% (wt/vol) yeast extract (Difco Laboratories, Detroit, Mich.). YEED agar plates contained 2% (wt/vol) agar (Difco) as a supplement. Yeast Nitrogen Base (YNB) (Difco) with 2% (wt/vol) glucose and 2% (wt/vol) agar (Difco), with amino acids and bases added as required, was used as a selective medium after transformation of *Saccharomyces cerevisiae* YKKB-13 and of *C. glabrata*. Agar plates containing 5-fluoroorotic acid (Toronto Research Chemicals, North York, Ontario, Canada) at 50 μg/ml were made for regeneration of the *ura3* genetic marker in YNB selective medium with uridine at 50 μg/ml. *Escherichia coli* DH5α (11) was used as a host for plasmid constructions and propagation and was grown on Luria-Bertani (LB) medium with ampicillin.

Susceptibility testing. Tests of susceptibility to azole antifungals were performed using the broth microdilution assay according to NCCLS protocol M27-A (21) using RPMI 1640 medium (Difco) and incubation at 35°C for 48 h. Endpoint readings were recorded with a microplate reader (Bio-Rad, Hercules, Calif.), and the azole concentration yielding at least 50% growth inhibition compared to growth in drug-free medium was defined as the MIC.

Susceptibility of the *C. glabrata* isolates and of *S. cerevisiae* strains containing *CgCDR1* and *CgCDR2* expression plasmids to different compounds was also tested qualitatively by spotting serial dilutions of yeast cultures onto complex YEED medium agar plates with different drug concentrations. This provides easy visualization of growth differences between different yeast strains. Each plate contained 15 ml of agar. Preliminary tests were performed to optimize drug concentrations in YEED plates so that growth differences between the different *S. cerevisiae* and *C. glabrata* strains used in this study could be observed. To perform the susceptibility tests, yeast strains were grown overnight at 30°C with constant shaking in YEED liquid medium. The cultures were diluted to 2 × 10⁷ cells per ml in 0.9% (wt/vol) NaCl. Five-microliter volumes of this suspension and serial dilutions of the yeast cultures were spotted onto each type of plate and incubated for 48 h at 30°C.

Isolation of *C. glabrata* RNA, genomic DNA, and mtDNA. Small-scale isolation of RNA and DNA from *C. glabrata* was performed as previously described (26). Mitochondrial DNA (mtDNA) was extracted from growing *C. glabrata* cultures by spheroplasting of cells and then sedimentation of mitochondria as described in reference (8).

Northern and Southern blot assays. Northern and Southern blot assays were carried out as described previously (26). ³²P-labeled DNA probes were generated by random priming (9) and added to the hybridization solution overnight. DNA probes in Southern and Northern blots were for *CgCDR1*, a 1.8-kb *XbaI*-*Bam*HI fragment from pNB126 described in reference (26). The *CgCDR2* probe was a 1-kb *Eco*RI fragment isolated from pDS521 (see below). The *CgURA3* probe was generated from genomic DNA by PCR as reported previously (26). Quantifications of Northern blots were performed by exposition of the hybridized membranes in an Instant Imager (Packard Instrument Company, Meriden,

Conn.). Signals were integrated by the software supplied by the manufacturer and normalized to the *CgURA3* probe (used as an internal standard).

Cloning of *CgCDR2* and *CgSNQ2*. Reverse-transcribed cDNA was prepared using the First Strand cDNA Synthesis Kit for reverse transcription (RT)-PCR from Roche Molecular Biochemicals (Rotkreuz, Switzerland) with total RNA prepared from *C. glabrata* strain DSY1041-HFAR (Table 1). This cDNA was used as the template for PCR amplification with primers PDR-GUESS R (5'-TGTTCVACATTTARACCTTCACCGSSAAC-3') and PDR-GUESS 3 (5'-AAATTYCAATGYGGGATAAYGCHACVAGR-3'). The design of these primers was based on comparisons with DNA sequences of the *S. cerevisiae* *PDR5*, *PDR10*, and *PDR15* genes and the *C. glabrata* *CgCDR1* gene and corresponding to the Walker A and B ATP-binding motifs of ABC transporters. The products obtained by RT-PCR, with an expected size of approximately 2 kb, were subcloned either into vector pCR2.1 (Invitrogen Corporation, Carlsbad, Calif.) or into *Eco*RI-*Hind*III-digested pBluescript (Stratagene) after restriction digestion of the same PCR products by the same enzymes. Both strategies ensured the cloning of all of the possible PCR products obtained. Individual clones were sequenced and revealed the presence of two distinct nucleotide sequences. The first one, which was similar to portions of *CgCDR1* and almost identical to a 2-kb portion of *PDH1* (19), was cloned into pCR2.1 (Genbank accession number AF251023). The second one, which was most similar to ABC transporter gene *SNQ2* from *S. cerevisiae*, was therefore named *CgSNQ2* (Genbank accession number AF251022) and was cloned into the *Hind*III-*Eco*RI sites of pBluescript.

Disruption of *CgCDR2*. pCR2.1, containing the *CgCDR2* gene cloned by RT-PCR, was digested with *Eco*RI, and a fragment of approximately 1 kb was cloned into the *Eco*RI site of pBluescript. The resulting plasmid, pDS521, was digested with *Bgl*II, which cuts within *CgCDR2*, and the 3.7-kb *Bam*HI-*Bgl*II fragment from pNKY51 (1) containing the *hisG-URA3-hisG* cassette was inserted at this site. The resulting plasmid, pDS547, was digested with *Eco*RI, and the 4.7-kb fragment containing the *hisG-URA3-hisG* cassette flanked by *CgCDR2* sequences was used to transform DSY1029. One of the Ura⁺ clones (DSY1612) was selected, and the correct integration of the disruption cassette was verified by Southern blotting (data not shown). The *ura3* genetic marker was regenerated by 5-fluoroorotic acid treatment of DSY1612. The *ura3* derivative of DSY1612 was used for the disruption of *CgCDR1* as described previously (26). The correct gene replacement of *CgCDR1* by the *hisG-URA3-hisG* disruption cassette was also verified (data not shown). The resulting double deletion (*CgCDR1 CgCDR2*) strain was named DSY1613.

Expression of *CgCDR1* and *CgCDR2* in *S. cerevisiae*. The *CgCDR1* and *CgCDR2* open reading frames (ORFs) were cloned into the *Hind*III site of pAAH5, which is a vector expressing ORFs under the control of the *ADH1* promoter (2). The *CgCDR1* and *CgCDR2* ORFs were amplified by high-fidelity PCR (Expand PCR; Roche Molecular Biochemicals) with primers to which *Hind*III sites were added. The following pairs of primers were for *CgCDR1* and *CgCDR2*, respectively (*Hind*III site underlined): 5'-GCGCAAGCTTACAATG TCTCTTGCAAGTGACAAGAAG-3' and 5'-GCGCAAGCTTTTATTTCTTG GCAAGTTTACCAGATTT-3'; 5'-GCGCAAGCTTACAATGG CAATTGGT

ATATATACTGGAACG-3' and 5'-GCGCAAGCTTCTAGAAGGGAATTAA CCTTCTAATAAAATTAACC-3'. Genomic DNA from clinical isolate DSY562 served as the template in the PCR. The *CgCDR1* and *CgCDR2* expression plasmids obtained (pDS646 and pDS647, respectively) were transformed into *S. cerevisiae* YKKB-13. Verification that a functional protein was being produced from the genes contained in each plasmid was that each plasmid conferred resistance to azole antifungal agents when transformed into *S. cerevisiae* YKKB-13.

Isolation of mitochondrial mutants of *C. glabrata*. *C. glabrata* strains were grown overnight in YEPD liquid medium and diluted 100-fold in 2 ml of fresh medium. Ethidium bromide (EtBr) was added from a sterile filtered stock solution to a final concentration of 20 µg/ml. The culture was grown overnight at 30°C, and an aliquot was plated on YEPD agar to isolate single colonies. After 2 days of incubation at 30°C, single colonies were selected and tested for their growth phenotypes on glycerol- and glucose-containing media. Colonies growing not on glycerol medium but on glucose medium were analyzed for the presence of mtDNA by staining with MitoTracker Green FM (see below) or by agarose gel electrophoresis. Mutants lacking mtDNA (considered rho⁰) were further selected.

Immunoblot assays. To allow detection of the proteins encoded by *CgCDR1* and *CgCDR2* (i.e., CgCdr1p and CgCdr2p) in yeast extracts, polyclonal antibodies against these proteins were raised in rabbits. The proteins for immunization were obtained in *E. coli* by fusion of the first 60 amino acids of each protein with glutathione *S*-transferase (GST). The GST fusion constructs were prepared by subcloning PCR fragments obtained using genomic DNA from *C. glabrata* DSY562 as the template. The following pairs of primers were used for *CgCDR1* and *CgCDR2*, respectively (*Bam*HI and *Eco*RI sites underlined): 5'-GCGCGG AATTCATGCTCTTGTCAAGTGACAAGAAGGA-3' and 5'-GCGCGGAATTC CAGGCAGAGTGTGTGTTCTTTCTTTTGTATG-3'; 5'-GCGCGGATCCAT GGAACACACCCGATGACTCTAGTGT-3' and 5'-GCGCGGAATTCCTCA ATGGCGCGCTGCCATCTGCGGGGGC-3'. The PCR fragments obtained were subcloned after restriction digestion with *Eco*RI and *Bam*HI into the same sites of the vector pGEX-2T (Amersham Pharmacia Biotech, Dübendorf, Switzerland) to allow in-frame fusions with GST. The purification of GST fusion proteins for raising polyclonal rabbit antibody was achieved by affinity chromatography of *E. coli* cell extracts on glutathione-agarose using standard procedures described by the manufacturer (Amersham Pharmacia Biotech).

These antibodies were used to detect, by immunoblot assay, CgCdr1p and CgCdr2p from cellular proteins of *C. glabrata* or *S. cerevisiae* transformed with expression plasmids. Cell extracts were prepared, and immunoblot assays were performed essentially as described by Sanglard et al. (27).

Measurement of total cytochrome content. Cytochromes *aa*₃, *b*, and *c* were measured in whole cells by difference spectra of reduced versus oxidized cells. *C. glabrata* strains were first grown in liquid YEPD overnight at 30°C, and equivalent cell densities (8 × 10¹⁰ cells) were pelleted by centrifugation. The pelleted cells were resuspended in 6 ml of phosphate-buffered saline (PBS) and distributed in separate 5-ml quartz cuvettes. After baseline correction, sodium dithionite and H₂O₂ were added to each cuvette and the difference spectra were recorded from 500 to 650 nm with a Lambda 18 spectrophotometer (Perkin-Elmer International, Rotkreuz, Switzerland).

Staining of mitochondria. *C. glabrata* isolates were grown overnight in liquid YEPD medium. Cells (2 × 10⁷) were washed twice with PBS and resuspended in 0.5 ml of PBS containing 0.5% (wt/vol) glucose. MitoTracker Green FM (Molecular Probes Inc., Eugene, Oreg.) was added at a final concentration of 100 nM, and the mixture was incubated at room temperature for 30 min. Cells were directly observed for green fluorescence with a Zeiss Axioplan microscope equipped with fluorescence filter set 13.

Fluorescence microscopy. Fluorescence microscopy and phase-contrast microscopy were performed with a Zeiss Axioplan microscope (Zeiss) equipped for epifluorescence microscopy with a 100-W high-pressure mercury bulb and Zeiss fluorescein-specific filter set 13. A Kappa DX30 digital camera with high resolution (Kappa Messtechnik GmbH, Gleichen, Germany) was used to record images, which were further processed using the computer program Adobe Photoshop 5.0 (Adobe Systems Incorporated, Mountain View, Calif.).

Other methods. Rhodamine accumulation was measured by flow cytometry as previously described (26).

Chemicals. Analytical-grade or equivalent chemicals were used in this study. Restriction enzymes and DNA-modifying enzymes were from Roche Molecular Biochemicals.

RESULTS

HFAR in *C. glabrata* is linked with upregulation of ABC transporter genes. We recently showed that deletion of ABC

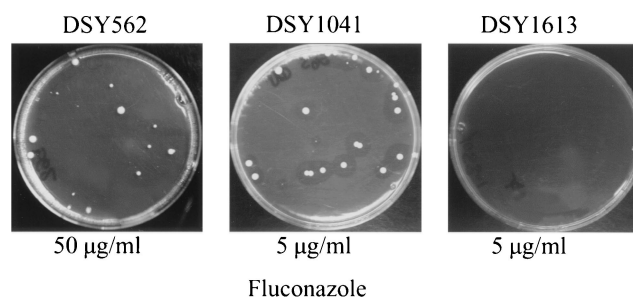


FIG. 1. HFAR in *C. glabrata* strains DSY562, DSY1041, and DSY1613 following growth on YEPD containing fluconazole. The inoculum size of DSY562 on the YEPD plate containing fluconazole at 50 µg/ml was 3.3×10^4 cells. The frequency of HFAR cells in three independent experiments was $3.2 \times 10^{-4} \pm 0.71 \times 10^{-4}$. Inoculum sizes for DSY1041 and DSY1613 were 4×10^4 and 5×10^4 cells, respectively. The frequency of HFAR cells from DSY1041 was similar to that obtained with DSY562. Incubation of the plates was for 4 days at 30°C. The fluconazole concentration used in YEPD is indicated for each plate.

transporter gene *CgCDR1* in an azole-resistant *C. glabrata* clinical isolate (DSY1041) resulted in increased susceptibility of the mutant to azole derivatives (26). We observed, however, that when DSY1041 was incubated on agar medium containing several azole derivatives, including fluconazole and itraconazole, several colonies could still appear from individual cells. Plating a high density of the mutant DSY1041 onto rich agar medium containing fluconazole at 5 µg/ml gave rise to fluconazole-resistant cells at frequencies ranging from 2×10^{-4} to 4×10^{-4} . These cells were referred to as HFAR cells (Fig. 1). HFAR cells could also be generated from azole-susceptible wild-type *C. glabrata* strain DSY562 at a frequency similar to that of DSY1041 but using agar medium containing as much as 50 µg of fluconazole per ml (Fig. 1). Azole MICs for HFAR derivatives and for their parents are shown in Table 2. Fluconazole MICs and itraconazole MICs for HFAR derivatives obtained from DSY562 and DSY1041 were higher than those of their respective parent strains. Furthermore, azole MICs for HFAR strains were found to be similar to those exhibited by azole-resistant clinical strain DSY565 (Table 2). To examine the mechanism of resistance operating in HFAR cells, accumulation of rhodamine 6G was first tested, since we showed previously that poor accumulation of this compound in azole-

TABLE 2. Azole MICs for *C. glabrata* isolates used in this study

<i>C. glabrata</i> strain	MIC (µg/ml) of:	
	Fluconazole	Itraconazole
DSY562	4	0.25
DSY565	>128	4
DSY562-HFAR ^a	>128	2
DSY562-rho ^b	>128	2
DSY1041	8	0.5
DSY1041-HFAR ^a	16	1
DSY1041-rho ^b	16	1
DSY1613	4	0.25
DSY1613-rho ^b	8	0.5

^a Strain obtained by HFAR.

^b Strain obtained by EtBr treatment.

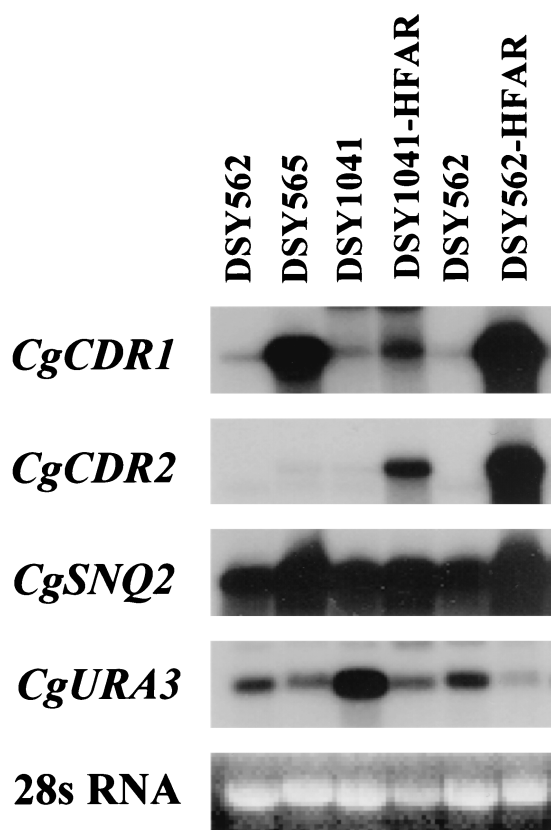


FIG. 2. Expression of ABC transporter genes in fluconazole-resistant isolates of *C. glabrata*. RNA was extracted from *C. glabrata* clinical isolates DSY562 and DSY565, from *CgCDR1* deletion mutant DSY1041, and from HFAR cells obtained with DSY562 and DSY1041. The Northern blot was probed sequentially with ^{32}P -labeled probes specific for all of the genes (*CgCDR1*, *CgCDR2*, *CgSNQ2*, and *CgURA3*) as indicated. The mRNA-hybridizing band detected in RNA of DSY1041-HFAR is probably due to cross-hybridization of the *CgCDR1* probe with *CgCDR2* mRNA, as discussed by Sanglard et al. (26). Due to variations in *CgURA3*-specific signals, EtBr-stained 28S RNA is shown to indicate that similar RNA quantities were loaded on the agarose gel. Using *CgURA3* signals for normalization, *CgCDR1* signals were increased 25- and 9-fold in DSY562-HFAR and DSY565, respectively, compared to those detected in parent strain DSY562.

resistant cells was linked with upregulation of multidrug transporter genes, especially *CgCDR1* (26). HFAR cells which arose from DSY1041 (DSY1041-HFAR) and DSY562 (DSY562-HFAR) accumulated 6.5- and 6-fold less rhodamine 6G than their azole-susceptible parents, respectively, suggesting that multidrug transporter genes could be upregulated in these strains. Expression of *CgCDR1* could only be examined in DSY562-HFAR, since this gene was deleted in the DSY1041-HFAR strain. A 25-fold relative increase in *CgCDR1* mRNA could be measured in DSY562-HFAR compared to DSY562. The increase in the *CgCDR1* mRNA signal in DSY562-HFAR was approximately 2.5-fold greater than that obtained for the same gene in azole-resistant clinical strain DSY565 (Fig. 2). The increase in azole MICs measured for DSY562-HFAR could therefore be attributed, at least in part, to the upregulation of *CgCDR1*.

Since *CgCDR1* was deleted in DSY1041 and since it was

possible to obtain HFAR cells from this strain, we considered that another or several other multidrug transporter genes were upregulated. The reduced accumulation of rhodamine 6G in DSY1041-HFAR was consistent with this hypothesis. To address this question, degenerate primers corresponding to a region conserved among yeast ABC transporter genes were used in a PCR carried out with reverse-transcribed mRNA from DSY1041-HFAR. From the expected PCR products of approximately 2 kb, two different sequences were obtained. One was similar to a portion of ABC transporter gene *SNQ2* from *S. cerevisiae*, whereas the other was similar to *CgCDR1* but identical to the *C. glabrata* ABC transporter gene *PDH1* reported recently (19) (see Materials and Methods for further details). These genes were named *CgSNQ2* and *CgCDR2*, respectively, in this work. The nucleotide sequence of *CgCDR2* cloned in this work was 98% similar to the comparable portion of *PDH1*. Labeled probes made from *CgSNQ2* and *CgCDR2* were hybridized to a Northern blot containing RNAs extracted from several *C. glabrata* strains. Whereas the expression of *CgSNQ2* was little affected by azole resistance, the intensity of the mRNA signal obtained with *CgCDR2* was increased in HFAR strains (Fig. 2). We calculated an up-to-100-fold increase in the *CgCDR2* mRNA signal in strain DSY562-HFAR compared to the signals obtained in strain DSY562. Therefore, of the two genes isolated by RT-PCR, only *CgCDR2* seemed important for the development of azole resistance. This view was confirmed by examination of *CgCDR2* expression in strain DSY1041-HFAR, in which *CgCDR1* was deleted (Fig. 2). In this strain, the mRNA signal corresponding to *CgCDR2* was increased 16-fold compared to that in azole-susceptible strain DSY1041. Therefore, it is likely that the azole resistance restored in DSY1041-HFAR was due, at least in part, to *CgCDR2* upregulation. However, the role of other, still uncharacterized, multidrug transporter genes in the HFAR phenomenon cannot be ruled out yet.

Deletion of *CgCDR2* in *C. glabrata*. To address the importance of *CgCDR2* in the acquisition of azole resistance by *C. glabrata*, this gene was deleted by targeted deletion in DSY1041. Southern blot hybridization confirmed that the deletion of *CgCDR2* had taken place at the correct genomic locus (data not shown). The resulting double deletion (*CgCDR1* *CgCDR2*) mutant was named DSY1613. The deletion of both *CgCDR1* and *CgCDR2* in DSY1613 resulted in the absence of full-length *CgCDR*-specific mRNAs (Fig. 3A) and, consistently, in the absence of detectable CgCdr1p and CgCdr2p (Fig. 3B). DSY1613 was more susceptible to azole derivatives than its parent DSY1041 when tested in media containing low concentrations of fluconazole, ketoconazole, and itraconazole (Fig. 4). More importantly, this strain was not capable of forming HFAR cells on a medium containing fluconazole at 5 $\mu\text{g}/\text{ml}$ under conditions in which DSY1041 formed HFAR cells (Fig. 1). Thus, it appears that both *CgCDR1* and *CgCDR2* are important for the in vitro acquisition of azole resistance in *C. glabrata*. Furthermore, since HFAR cells were obtained from DSY1041, *CgCDR2* upregulation can compensate for the absence of *CgCDR1* and can contribute to decreased azole susceptibility.

The level of azole resistance obtained by *CgCDR2* upregulation, however, seemed lower than that obtained by *CgCDR1*, since no HFAR cells could be isolated in a medium containing fluconazole at a concentration of 50 $\mu\text{g}/\text{ml}$ (data not shown).

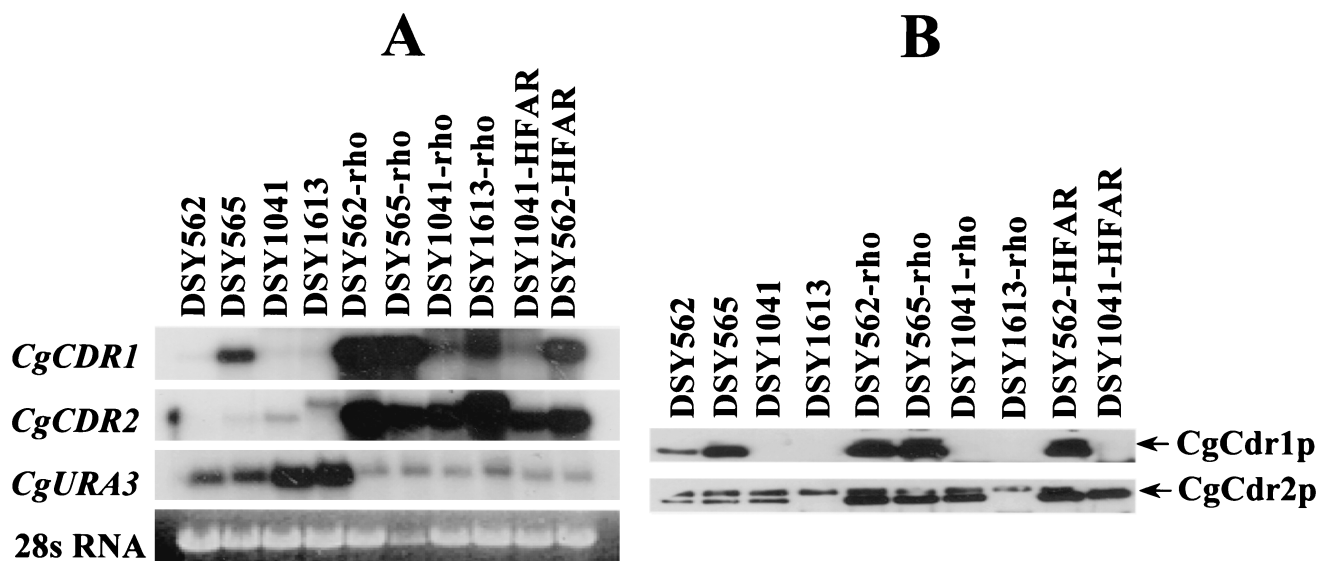


FIG. 3. Expression of multidrug efflux transporters in *C. glabrata* isolates with in vitro-acquired azole resistance. (A) Northern blot analysis. Approximately 5 μ g of total RNA from each indicated yeast strain was loaded on the agarose gel. The Northern blot was hybridized sequentially with each 32 P-labeled probe. The *CgCDR2*-specific signal from DSY1613 has a larger size than that from DSY1041 and probably corresponds to an aberrant RNA product. The Northern blots were revealed by exposure of Fuji XAR film at -80°C . Normalized signals for *CgCDR1*, compared to those detected in DSY562, were increased 7-, 62-, 67-, and 90-fold in DSY565, DSY562-rho, DSY565-rho, and DSY562-HFAR, respectively. Normalized signals for *CgCDR2*, compared to those detected in DSY562, were increased 1.7-, 1-, 68-, 40-, 22-, 26-, and 80-fold in DSY565, DSY1041, DSY562-rho, DSY565-rho, DSY1041-rho, DSY1041-HFAR, and DSY562-HFAR, respectively. (B) Immunodetection of CgCdr1p and CgCdr2p in cellular extracts. A 10- μ g sample of total protein was loaded on a sodium dodecyl sulfate–10% (wt/vol) polyacrylamide gel and separated by electrophoresis. Western blots were incubated separately with CgCdr1p and CgCdr2p antisera, and signals were revealed by chemiluminescence on Fuji XAR film. Absence of signals in protein extracts from DSY1041 and DSY1613 is consistent with the deletion of *CgCDR1* and *CgCDR2* in these strains.

To test this hypothesis, *CgCDR1* and *CgCDR2* were expressed under the control of the same *ADHI* promoter in azole-hypersusceptible *S. cerevisiae* strain YKKB-13, in which the ABC transporter gene *PDR5* has been deleted. Figure 5 shows that DSY1696, in which *CgCDR1* was expressed, was still able to grow in a medium containing fluconazole at 5 μ g/ml, whereas DSY1694 expressing *CgCDR2* could only grow at lower drug concentrations. The same relationship was observed in a medium containing cycloheximide, which is a known substrate for *CgCDR1* (26). These results confirmed our above-mentioned hypothesis that *CgCDR1* has a higher capacity than *CgCDR2* for the development of fluconazole resistance.

The growth defect of HFAR cells is paralleled by loss of mitochondria. Upon repeated subculture of DSY562-HFAR in fluconazole-free medium, the fluconazole MIC for this strain dropped from >128 μ g/ml (subculture A) to 64 μ g/ml (subculture P), which corresponds approximately to a span of 200 generations after the start of the experiment. Figure 6A shows that upregulation of both *CgCDR1* and *CgCDR2* was still detected for up to 200 generations in drug-free medium. Quantification of *CgCDR1* and *CgCDR2* mRNA signals normalized with the *CgURA3* probe, however, revealed a tendency to decrease with increasing subculture. In DSY562-A, the signals for *CgCDR1* and *CgCDR2* mRNAs were increased 60- and 90-fold, respectively, compared to those detected in DSY562, and the increases declined to 10- and 5-fold in DSY562-P. Increases in normalized mRNA signals for *CgCDR1* and *CgCDR2* in DSY562-P compared to DSY562 were similar to those obtained in azole-resistant clinical strain DSY565. The correla-

tion between multidrug transporter gene expression and fluconazole MICs could not be well established because the fluconazole concentration in the MIC test was limited to 128 μ g/ml. However, although multidrug transporter gene expression declined with repeated subculture but was still comparable to the expression observed in azole-resistant clinical isolates, we considered that azole resistance could be maintained in HFAR cells under drug-free culture conditions. When growth of HFAR cells was recorded, optical densities did not reach the values measured in cultures of parent strains DSY562 and DSY565 in late lag phase (Fig. 6B). Subtle differences were, however, noticed in the initial growth rate of HFAR cells, depending on the degree of subculture. While early subcultures showed initial slow growth (for example, subculture DSY562-A or DSY562-D), subsequent subcultures (DSY562-L or DSY562-P) grew faster in their log phase. This trend closely followed the relative increases in *CgCDR1* and *CgCDR2* expression, where both genes, being highly upregulated in first subculture, were gradually less upregulated in subsequent subculture. The cell densities of DSY562-HFAR strains obtained in late log phase, however, remained almost identical. The restoration of the measured initial growth rate seemed to be related to the decrease in *CgCDR1* and *CgCDR2* expression, as shown in Fig. 6A. These results suggest that high expression levels of these multidrug transporter genes could have adverse effects, the consequence of which results in reduced growth rate.

When tested for the ability to grow on different carbon sources, HFAR cells did not grow on ethanol and arabinitol,

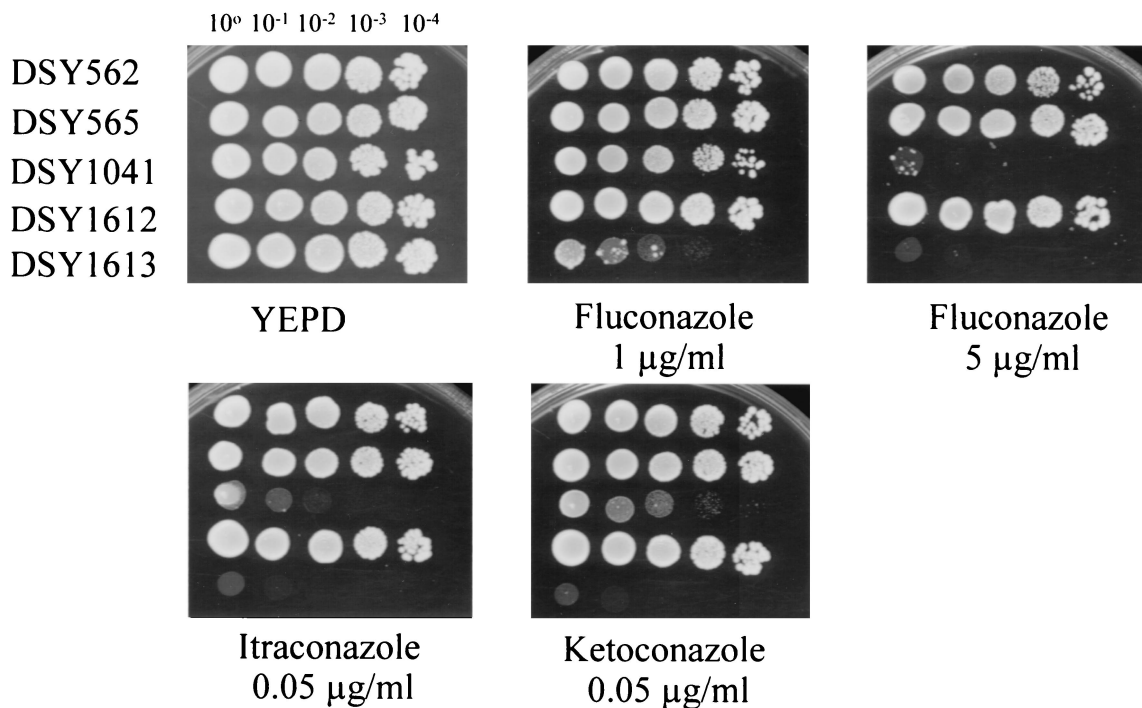


FIG. 4. Susceptibility of *C. glabrata* multidrug efflux transporter mutants to azole derivatives. Each yeast strain is indicated at the left. Azole derivatives were added to YEPD medium at the indicated concentrations. Plates were incubated for 48 h at 30°C.

which are nonfermentable carbon sources (data not shown). The lack of growth on these carbon sources suggested alterations in the mitochondrial respiratory capacity of these yeasts. A similar phenomenon is known in *S. cerevisiae*, and cells with a respiratory deficiency are called petite mutants. These cells often lack mtDNA. Since the reduced respiratory capacity detected in *C. glabrata* could probably be attributed to a loss of

mitochondria, staining with the mitochondrion-specific fluorescent dye MitoTracker Green FM was applied to *C. glabrata* in growing cultures. Figure 7 shows positive staining in respiratorily competent (i.e., rho⁺) cells, whereas weak or absent staining was seen in HFAR cells. The absence of mtDNA was verified in HFAR cells by subjecting their mitochondrial cell fractions to agarose gel electrophoresis, and no mtDNA bands

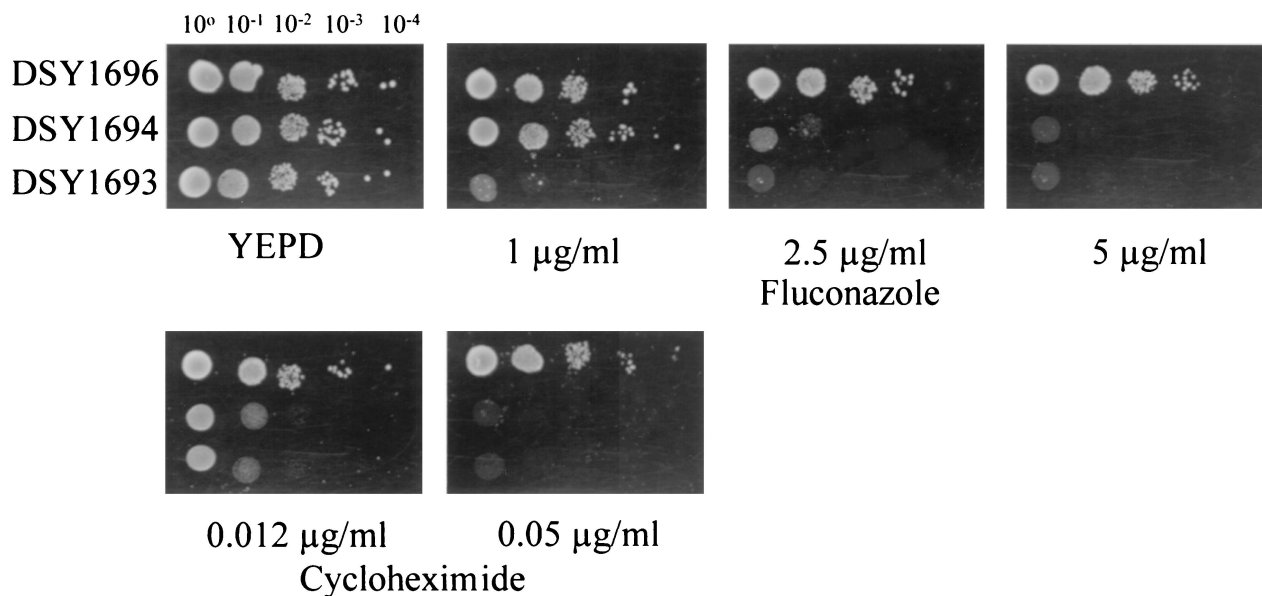


FIG. 5. *CgCDR1* and *CgCDR2* expression in *S. cerevisiae*. Susceptibility tests of yeast transformants were performed with fluconazole and cycloheximide at the indicated concentrations. Plates were incubated for 48 h at 30°C.

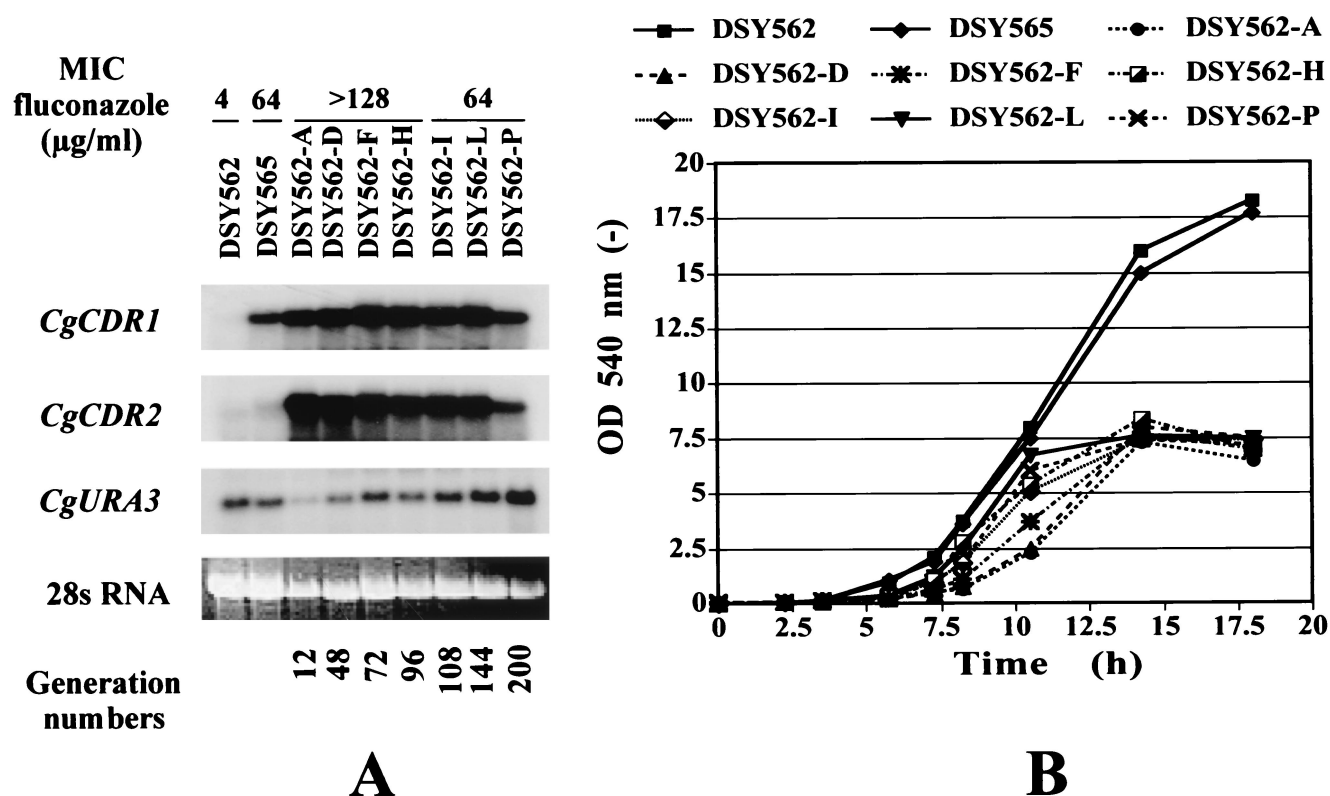


FIG. 6. Maintenance of azole resistance of HFAR cells in drug-free medium. (A) Expression of *CgCDR1* and *CgCDR2* in DSY562-HFAR in drug-free YEPD medium over subcultures A (no. 1) to P (no. 16). Intensities of *CgCDR1* and *CgCDR2* signals were normalized to those obtained with the *CgURA3* probe. Normalized signals for *CgCDR1*, compared to those detected in DSY562, were increased 60-, 40-, 31-, 35-, 26-, 23-, and 8-fold in DSY562-A to -P, respectively, and 12-fold in DSY565. Normalized signals for *CgCDR2*, compared to those detected in DSY562, were increased 90-, 33-, 22-, 22-, 18-, 15-, and 5-fold in DSY562-A to -P, respectively, and 2.5-fold in DSY565. (B) Growth of clinical isolate DSY562, DSY565 (azole-resistant), and DSY562-HFAR subcultures (A to P) in YEPD.

could be stained with EtBr (data not shown). The loss of mitochondria in these cells can therefore account for their above-mentioned growth defect. Petite mutants can be easily obtained in *S. cerevisiae* by treatment with EtBr. We applied this technique to *C. glabrata* and obtained cells with the petite characteristics. These cells could not be stained with MitoTracker Green FM (Fig. 7) and failed to reveal mtDNA banding on gel electrophoresis analysis (data not shown), as in the case of HFAR cells. Petite mutants obtained by EtBr treatment were therefore considered ρ^0 according to the nomenclature accepted for *S. cerevisiae*. These cells were referred as to DSY- ρ strains in this work. Although generated by different chemical treatments, HFAR and ρ^0 cells possess similar characteristics. First, when total cytochrome content was measured in whole cells of HFAR and ρ^0 isolates by difference spectra, no absorption peak could be measured corresponding to those of cytochromes *aa₃* and *b*, which are gene products encoded by mtDNA (data not shown). Second, the azole MICs obtained for HFAR and ρ^0 strains were similar. As summarized in Table 2, the fluconazole and itraconazole MICs for DSY562-HFAR and DSY562- ρ were >128 and 2 µg/ml, respectively, whereas those for DSY1041-HFAR and DSY1041- ρ were 16 and 1 µg/ml. It is remarkable that DSY- ρ derivatives obtained by EtBr treatment acquired azole resistance without previous exposure to these antifungals.

Depending on the genetic background used to generate ρ^0 derivatives, variable degrees of azole resistance were obtained. Figure 8 shows, for example, that DSY562- ρ could grow on a medium containing fluconazole at up to 50 µg/ml, whereas DSY1041- ρ could only grow distinctly in a medium containing the same drug at only 2.5 µg/ml. Interestingly, the double mutant (*CgCDR1 CgCDR2*) strain DSY1613 rendered ρ^0 by EtBr treatment (DSY1613- ρ) acquired resistance to fluconazole. Azole resistance obtained in DSY1613- ρ was only visible when a low fluconazole concentration (i.e., 1 µg/ml) was present in YEPD plates. As shown in Fig. 8, DSY1613- ρ grew well at this drug concentration whereas parent strain DSY1613 grew weakly in the same medium.

The ρ^0 cells accumulated less rhodamine 6G than their wild-type parents, and the acquisition of this azole resistance was as stable as in the case of HFAR cells (data not shown). The expression of ABC transporter genes was tested in different ρ^0 cells with specific genetic backgrounds. *CgCDR1* and *CgCDR2* were upregulated in these cells compared to ρ^+ cells, to levels similar to those measured in HFAR cells (Fig. 3A). For example, *CgCDR1*-normalized mRNA signals were elevated 90- and 62-fold in DSY652- ρ and DSY562-HFAR cells, respectively, while *CgCDR2*-normalized mRNA signals were increased 80- and 68-fold in the same strains. Accordingly, *CgCdr1p* and *CgCdr2p* levels were higher in ρ^0 and

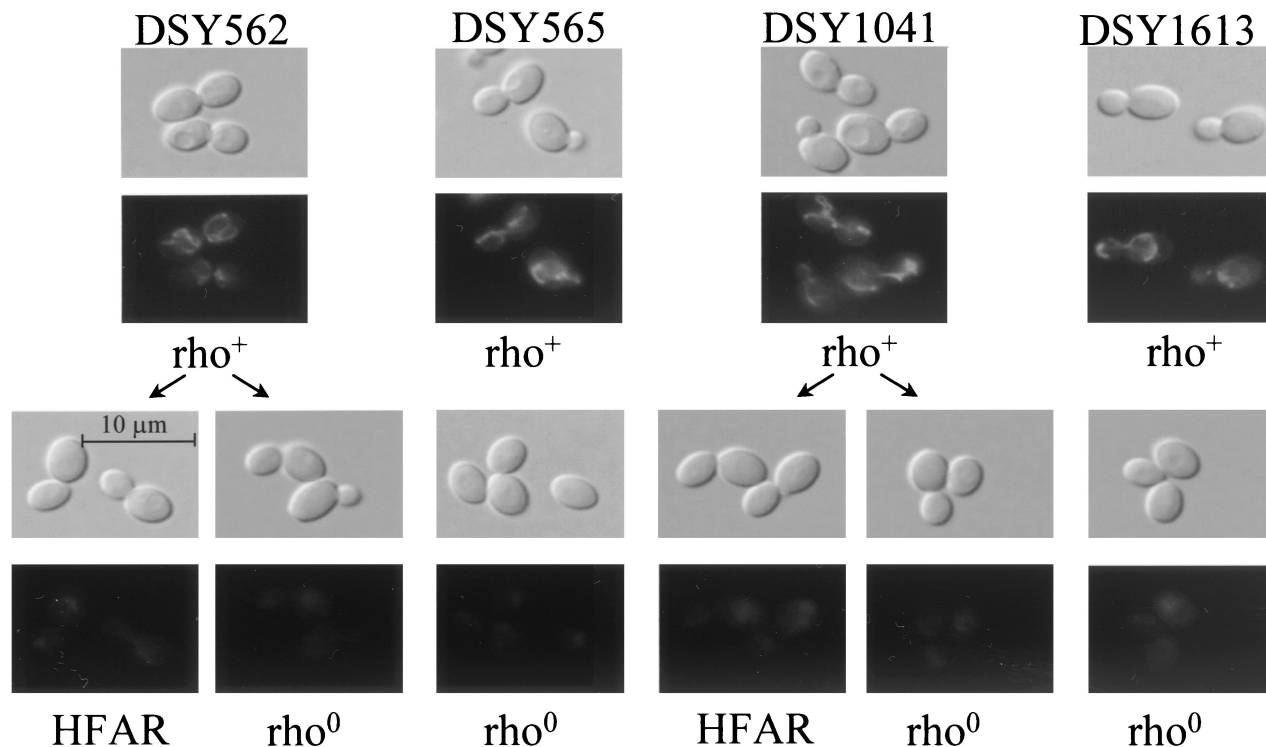


FIG. 7. Staining of mitochondria in *C. glabrata*. Strains DSY562, DSY565, DSY1041, and DSY1613 are respiratorily competent and therefore were designated ρ^+ . From each of these strains, HFAR cells obtained by fluconazole exposure or ρ^0 cells obtained by EtBr treatment were incubated with MitoTracker Green FM and examined by either phase-contrast (top rows) or epifluorescence (bottom rows) microscopy.

HFAR derivatives than in ρ^+ cells, except in cells in which the genes encoding these proteins were deleted (Fig. 3B).

DISCUSSION

We recently showed how *CgCDR1* could contribute to azole resistance in *C. glabrata* clinical isolates (26). In this study, we further detailed the molecular basis of azole resistance in this yeast species by exploring the role of another ABC transporter gene, *CgCDR2*, in this phenomenon. We isolated *CgCDR2*, which is identical to *PDH1* (19), through observations which revealed the possible expression of other multidrug transporter genes in a *CgCDR1* deletion strain exposed in vitro to fluconazole. The results shown in this study indicate that the regulatory system of these transporters might be quite complex. On the one hand, while the upregulation of *CgCDR1* is significant in azole-resistant clinical isolate DSY565, *CgCDR2* is only moderately expressed in the same strain. On the other hand, when azole resistance is established by HFAR or EtBr treatment from an azole-susceptible strain, both *CgCDR1* and *CgCDR2* are upregulated to high levels. This suggests that one or several regulatory systems should exist in *C. glabrata* enabling the differential regulation of these genes. Both genes possess in their promoter regions matching the consensus for Pdr1p-Pdr3p binding sites (15); therefore, one can expect that they can be controlled coordinately by Pdr1p-Pdr3p-like transcription factors. In the light of the results obtained here, it is likely that other regulatory elements assist the differential expression of *CgCDR1* and *CgCDR2*.

C. glabrata ABC transporter gene *PDH1*, reported by Miyazaki

et al. (19), has a sequence which is almost identical to that of *CgCDR2* in the region corresponding to the PCR-amplified fragment. Miyazaki et al. (19) reported that *PDH1* was upregulated up to fourfold in *C. glabrata* clinical strains resistant to fluconazole. In this study, slight upregulation of the same gene could be detected in azole-resistant clinical isolate DSY565. We examined the expression of *CgCDR2* in additional azole-susceptible or resistant clinical strains of *C. glabrata* and observed that this gene was also expressed at moderate levels. Upregulation of *CgCDR2* was noticed in a few azole-resistant strains, whereas *CgCDR1* upregulation was always clearly manifested (D. Sanglard and D. Calabrese, unpublished data). Further comparisons of the two transporters are under way. The data presented here favor the hypothesis that *CgCDR1* is more potent in its ability to confer azole resistance than is *CgCDR2*. First, HFAR cells derived from wild-type *C. glabrata* strains could be obtained on medium containing a high fluconazole concentrations (50 $\mu\text{g}/\text{ml}$; Fig. 1), whereas they could be obtained with DSY1041 (the *CgCDR1* deletion strain) only on medium with a lower fluconazole concentration (5 $\mu\text{g}/\text{ml}$; Fig. 1). Second, the heterologous expression of *CgCDR1* and *CgCDR2* in *S. cerevisiae* showed that *CgCDR1* expression resulted in higher fluconazole resistance levels than in the case of *CgCDR2* expression (Fig. 5). It is already known that ABC transporter genes with high similarity (for example, the *C. albicans* *CDR1* and *CDR2* genes) differ in the ability to confer resistance to the same drugs (28). Whether this effect is due to altered substrate specificity or reduced drug efflux capability has still not been precisely determined and deserves further investigation.

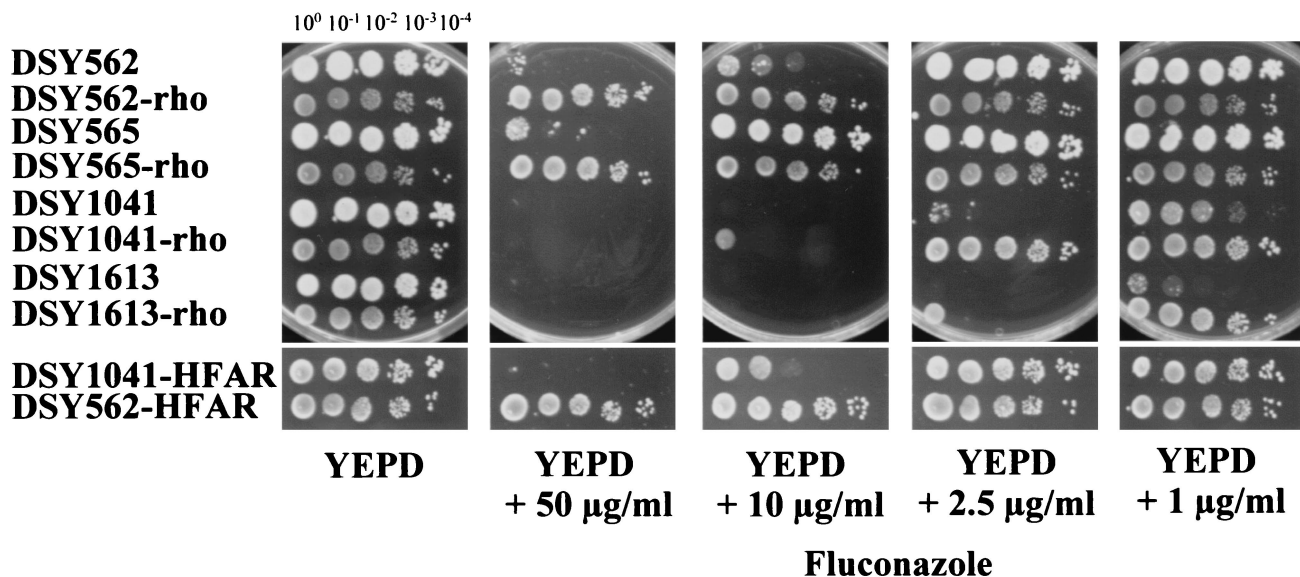


FIG. 8. Mitochondrial loss is linked to the acquisition of azole resistance in *C. glabrata*. Wild-type, HFAR, and rho⁰ cells were spotted in serial dilutions onto YEPD agar and fluconazole-containing YEPD medium. Plates were incubated for 48 h at 30°C.

Isolation of azole-resistant *C. glabrata* strains using miconazole has been reported, but no growth defects were observed in the azole-resistant strains obtained (23). In this work, we established a link in *C. glabrata* between loss of mitochondria and acquisition of azole resistance (HFAR) mediated at least by the ABC transporter genes *CgCDR1* and *CgCDR2*. There are discrepancies between the HFAR in *C. glabrata* described here and the phenomenon of heteroresistance reported in *C. neoformans* (20) and more recently in *C. albicans* (18). Heteroresistance implies that, from a single progenitor, different reversible drug resistance phenotypes can be obtained which are reflected by different drug MICs. In the present work, HFAR cells appeared from a single progenitor with similar resistance phenotypes and with uniform and irreversible mitochondrial loss. Thus, it is reasonable to distinguish HFAR from heteroresistance. It is likely that loss of mitochondria in *C. glabrata* will affect the expression of other genes, some of which are able to play a role in azole resistance. A good candidate could be the *C. glabrata* *ERG11* gene, the upregulation of which was coupled with azole resistance in some *C. glabrata* strains (16). In this work, it is striking that *CgURA3* expression was decreased in HFAR and rho⁰ mutant strains, although identical RNA quantities were loaded in the Northern blots of Fig. 2, 3, and 6. Expression studies on a genome-wide scale would be very helpful in determining the number of genes with altered expression upon loss of mitochondria. Recently, the acquisition of azole resistance by mitochondrial loss in *C. glabrata* was reported by Defontaine et al. (8); however, the authors of that study did not work out the molecular mechanisms behind this observation. One important question resulting from both the work of Defontaine et al. (8) and ours is the relevance of this phenomenon to clinical situations. One can expect that by being exposed to azole antifungals, azole-resistant *C. glabrata* strains would appear by HFAR and therefore be devoid of mitochondria. Under azole-selective pressure, these HFAR strains would have a clear advantage over still susceptible strains. However, due to their defective respiratory capacity, HFAR strains may be not

as competitive as wild-type cells under the environmental conditions of the host. If the antifungal selection is removed in a patient, HFAR cells may be very rapidly overgrown by other, more growth-competent strains. It would be interesting to test in animal models or in patients if HFAR cells could be detected when azole treatment is initiated and if these cells remain present after drug removal. Data recently reported by Bouchara et al. (6) reveal that *C. glabrata* mitochondrial mutants were obtained from stool samples taken from a bone marrow transplant patient undergoing fluconazole therapy, thus showing the potential relevance of this phenomenon to clinical situations.

Loss of mitochondria is a phenomenon well described in *S. cerevisiae* which can arise spontaneously or in response to DNA-targeting drugs (7). It is coupled with known phenotypes, one of them being growth deficiency in nonfermentable carbon sources. *S. cerevisiae* belongs to the group of petite-positive yeasts, for which loss of mitochondrial functions is not lethal. The mtDNA encodes essential functions for some other yeasts, and these yeasts are called petite negative. *C. glabrata* undoubtedly belongs to the group of petite-positive organisms. The close evolutionary relatedness between *C. glabrata* and *S. cerevisiae* helps to explain this characteristic. The fact that rho⁰ mutants of this yeast could be obtained by using an antifungal agent (i.e., fluconazole) other than a DNA-targeting drug has not been often reported. The capacity to form HFAR cells upon fluconazole exposure is not too surprising, since the upregulation of ABC transporter genes, the products of which use fluconazole as substrates, is one of the consequences of mitochondrial loss. With convenient EtBr treatment inducing mitochondrial loss in *C. glabrata*, it has been possible to isolate rho⁰ mutants of strain DSY1613. Although this yeast does not possess functional *CgCDR1* and *CgCDR2* genes, it was possible to observe acquisition of a slight degree of azole resistance in the DSY1613-rho derivative (Fig. 8). DSY1613-rho cells reduced their accumulation of rhodamine 6G to 60% compared to DSY1613 cells (data not shown) and therefore we considered this result a consequence of upregulation of one or sev-

eral putative additional multidrug transporter genes which could also belong to the ABC transporter family. This is a likely explanation given that a multiplicity of ABC transporter genes has been documented in other yeast species. We are currently attempting the cloning of a putative transporter expressed in DSY1613-rho cells by the RT-PCR strategy outlined above. The results obtained in this study can also help to define other conditions for the generation of azole-resistant derivatives in *C. glabrata*. Exposing *C. glabrata* to fluconazole-containing media with nonfermentable carbon sources will allow us to obtain azole-resistant strains with mitochondria, since these organelles are required for the assimilation of these carbon sources. Such strains might be more comparable to respiratorily competent clinical isolates that are azole resistant. The analysis of such mutants is in progress in our laboratory.

In summary, we have shown here that *C. glabrata* exposed to fluconazole in vitro has the ability to acquire ABC transporter-mediated resistance through the loss of mitochondrial functions. Preliminary data obtained with *S. cerevisiae* mitochondrial mutants revealed a similar relationship (D. Sanglard, unpublished data). Azole resistance as a consequence of mitochondrial loss has not been reported in other yeasts pathogenic for humans until now. It might not be possible to address this question for some yeast species that are a priori petite negative. The respiratory capacity of these yeasts might, however, be transiently inhibited by specific inhibitors and their effects on azole resistance and expression of multidrug resistance genes could be investigated. Among yeast pathogens, mitochondrial mutants have been described only in *C. albicans* so far (3, 10, 24), despite the fact that *C. albicans* was considered a petite-negative yeast (17). Interestingly, *C. albicans* mitochondrial mutants are resistant to the antimicrobial peptide histatin 5 (10). It might be interesting to test the azole susceptibility of mitochondrial mutants of *C. albicans*.

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