The ATP Binding Cassette Transporter Gene CgCDR1 from
*Candida glabrata* Is Involved in the Resistance of Clinical
Isolates to Azole Antifungal Agents

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The resistance mechanisms to azole antifungal agents were investigated in this study with two pairs of *Candida glabrata* clinical isolates recovered from two separate AIDS patients. The two pairs each contained a fluconazole-susceptible isolate and a fluconazole-resistant isolate, the latter with cross-resistance to itraconazole and ketoconazole. Since the accumulation of fluconazole and of another unrelated substance, rhodamine 6G, was reduced in the azole-resistant isolates, enhanced drug efflux was considered as a possible resistance mechanism. The expression of multidrug efflux transporter genes was therefore examined in the azole-susceptible and azole-resistant yeast isolates. For this purpose, *C. glabrata* genes conferring resistance to azole antifungals were cloned in a *Saccharomyces cerevisiae* strain in which the ATP binding cassette (ABC) transporter gene *PDR5* was deleted. Three different genes were recovered, and among them, only *C. glabrata* CDR1 (*CgCDR1*), a gene similar to the *Candida albicans* ABC transporter CDR genes, was upregulated by a factor of 5 to 8 in the azole-resistant isolates. A correlation between upregulation of this gene and azole resistance was thus established. The deletion of *CgCDR1* in an azole-resistant *C. glabrata* clinical isolate rendered the resulting mutant (*DSY1041*) susceptible to azole derivatives as the azole-susceptible clinical parent, thus providing genetic evidence that a specific mechanism was involved in the azole resistance of a clinical isolate. When *CgCDR1* obtained from an azole-susceptible isolate was reintroduced with the help of a centromeric vector in *DSY1041*,azole resistance was restored and thus suggested that a trans-acting mutation(s) could be made responsible for the increased expression of this ABC transporter gene in the azole-resistant strain. This study demonstrates for the first time the determinant role of an ABC transporter gene in the acquisition of resistance to azole antifungals by *C. glabrata* clinical isolates.

Patients with advanced human immunodeficiency virus infection develop opportunistic infections due to the decrease in their immunity. Oropharyngeal candidiasis (OPC) caused by *Candida albicans* is a very common opportunistic infection in these patients and is treated mainly with azole antifungal agents, particularly with fluconazole. Treatment failures have been observed following the repeated use of this agent in relapses of OPC (21, 37, 45, 55). Different laboratories have reported that *C. albicans* isolates sampled sequentially during fluconazole treatment showed decreased susceptibility to fluconazole compared to that of the isolates sampled at the time of the first episode of infection (6, 30, 41, 53). Clinical resistance to fluconazole has been correlated with in vitro resistance of the yeasts recovered from patients undergoing antifungal therapy (54). This phenomenon has been also documented in other yeast species, including *Candida glabrata* (4, 16), *Candida tropicalis* (28), and *Candida krusei* (57, 58), and in Cryptococcus neoformans (27).

The increasing number of azole-resistant isolates recovered in many institutions during the past decade has motivated studies with the aim of understanding their mechanisms of resistance at the molecular level. Until now, *C. albicans* isolates have provided a major source for the discovery of mechanisms of azole resistance. Recent findings have shown that increased azole efflux is an important mechanism of resistance in yeast clinical isolates. In azole-resistant *C. albicans* isolates, increased azole efflux has been correlated with the upregulation of multidrug efflux transporter genes from two distinct families, the ATP binding cassette (ABC) transporters (CDR1 and CDR2) and the major facilitators (*C. albicans* *MDR1*) (3, 27, 30, 50, 53, 60). Deletion of CDR1 in *C. albicans* leads to azole hypersusceptibility and increased fluconazole accumulation (51). Decrease in azole affinity of the target enzyme of these antifungals, i.e., the cytochrome P450 lanosterol demethylase (called CYP51A1, or ERG11), has also been explained at the molecular level. Mutations in the genes encoding CYP51A1 (*CYP51A1*) have been detected in azole-resistant yeasts. These mutations resulted, in some cases, in amino acid substitutions with the probable effect of altering the binding properties of azoles and thus contributing to a decrease in azole susceptibility in clinical yeast isolates (49). Another mechanism of azole resistance originates from alterations in the ergosterol biosynthesis pathway, often resulting in the absence of ergosterol. This feature renders cells affected by this mechanism cross-resistant to amphotericin B. A few *C. albicans* clinical isolates possess this property and have been found to accumulate 14α-methylergosta-8,24(28)-dien-3β,6α-diol, which is indicative of a defect in Δ 5,6 sterol desaturase (24, 35). The above-mentioned resistance mechanisms can combine with each other in *C. albicans* and complicate the analysis of such isolates, since it is difficult to establish the role of an individual mechanism in the decrease in azole susceptibility (48). Dissection of resistance mechanisms by the use of genetics in *C. albicans* clinical...
isolates resistant to azole antifungal agents has not been reported yet. This lack of important information arises from the difficulties of developing reliable genetic systems for this diploid organism.

Historically, \textit{C. albicans} accounted for 70 to 80\% of organisms isolated in patients infected by fungal species. However, recent data report a population shift toward non-\textit{C. albicans} yeast species, such as \textit{C. glabrata}, \textit{C. tropicalis}, or \textit{C. krusei} (15). Among the non-\textit{C. albicans} species, \textit{C. glabrata} has emerged as an important nosocomial pathogen. Berrouane et al. (7) reported that among \textit{Candida} species, the proportion of \textit{C. glabrata} infections in the Iowa University Hospitals from 1988 to 1994 increased significantly, while it remained unchanged for other yeast species and even decreased for \textit{C. albicans}. Other investigators have noted similar increases in the frequency of infections caused by \textit{C. glabrata}, mostly in conjunction with the use of azoles (34, 39, 40). We also observed that \textit{C. glabrata} was often recovered from cultures originating from AIDS patients with OPC. \textit{C. glabrata} is known to be less susceptible to fluconazole than most of the \textit{C. albicans} fluconazole-susceptible isolates. Rex et al. (43) reported that the minimal inhibitory concentration inhibiting 50\% of the yeast population investigated (MIC\textsubscript{50}) of fluconazole for 31 \textit{C. glabrata} isolates was 16 \textmu g/ml, while the MIC\textsubscript{50} was 0.25 \textmu g/ml for 129 \textit{C. albicans} isolates. In several patients who responded poorly to fluconazole therapy, we noticed that \textit{C. glabrata} isolates could persist and that their susceptibility to azoles was decreased. Since mechanisms of resistance to azoles have been less intensively investigated in non-\textit{C. albicans} species such as \textit{C. glabrata}, we addressed here the molecular basis of resistance in two pairs of isolates taken from two different AIDS patients with documented azole antifungal treatment failure. We first isolated \textit{C. glabrata} azole resistance genes by complementation of hypersensitivity of a \textit{Saccharomyces cerevisiae} ABC transporter mutant. From the three different azole resistance genes isolated, only \textit{CgCDR1}, which resembles the \textit{C. albicans} ABC transporter \textit{CDR} genes, was upregulated in the azole-resistant \textit{C. glabrata} isolates from these two patients. By introducing a genetic marker in an azole-resistant clinical isolate, not only could evidence for the participation of this gene in azole resistance be obtained, but the nature of the mutation or mutations implicated in the upregulation of \textit{CgCDR1} could be predicted.

### 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 yeast extract-peptone-dextrose (YPD) complex medium containing 2\% glucose, 1\% Bacto peptone (Difco Laboratories, Detroit, Mich.), and 0.5\% yeast extract (Difco). YEPD agar plates contained 2\% agar (Difco) as a supplement. Yeast nitrogen base (YNB [Difco]) with 2\% glucose and 2\% agar (Difco) with appropriate amino acids and bases was used as a selective medium after transformation of \textit{S. cerevisiae} YKKB-13 and \textit{C. glabrata}.

#### Fluconazole and rhodamine 6G accumulation.

Fluconazole accumulation testing was performed in duplicate with \textsuperscript{1}H-labelled fluconazole (Amersham Life Science, Little Chalfont, Buckinghamshire, United Kingdom) as described previously (53), but with a single incubation time of 20 min. Rhodamine 6G (Sigma, Fluka Chemie AG, Buchs, Switzerland) accumulation testing was performed with yeast cells grown to the logarithmic phase in 14 ml of sterile polystyrene tubes with 2 ml of YEPD at 30\°C under constant agitation. Rhodamine 6G labelling of cells was performed in 1 ml of YEPD with \textsuperscript{10} cells and containing 10 \textmu M rhodamine 6G. The mixture was incubated for 30 min at 30\°C, after which it was stopped by placing the tubes on ice. These conditions have been optimized for minimal incubation time and maximal rhodamine 6G accumulation (data not shown). The reaction mixture was then diluted 40-fold in cold sterile 0.1 M phosphate-buffered saline (PBS) at pH 7.0 and then directly subjected to flow cytometry in a FACSscan fluorescence-activated cell sorter (FACS) (Becton Dickinson, San Jose, Calif.). Fluorescence was measured at an excitation wavelength of 488 nm and an emission wavelength of 515 nm (F1 detector). The sheath fluid was Isotone II. Data were acquired for 1,500 cells with the FACSscan Lys II software. Rhodamine 6G efflux was determined with \textsuperscript{10} cells previously loaded by incubation with 10 \textmu M rhodamine 6G at 30\°C in YEPD. Cells were washed three times with YEPD medium at 4\°C to remove excess rhodamine 6G, and efflux was started by incubation at 30\°C in the same medium. The decrease in fluorescence of loaded cells was then recorded at regular time intervals.

#### Drug susceptibility tests.

Tests of susceptibility to azole antifungals were performed by broth microdilution assay according to the National Committee for Clinical Laboratory Standards (NCCLS) protocol M27-A (33) with 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 the growth in drug-free medium was defined as the MIC. Amphotericin B susceptibility was measured according to growth in Antibiotic Medium 3 broth (Difco) as recommended previously (42).

Susceptibility to different compounds of the \textit{C. glabrata} isolates and of \textit{S. cerevisiae} strains containing \textit{C. glabrata} drug resistance genes was also tested qualitatively by spotting serial dilutions of yeast cultures onto complex YEPD medium agar plates. This provides an easy visualization of growth differences between different yeast strains. Since \textit{S. cerevisiae} does not grow well in the RPMI medium described in the NCCLS protocol M27-A, the use of the qualitative plate assay for drug susceptibility is more adequate. The following drugs were solubilized in dimethyl sulfoxide: ketoconazole and itraconazole (Janssen Pharmaceuticals, Beerse, Belgium), 4-nitroquinoline-N-oxide (Sigma), and benomyl (Riedel-de-Haen, Seelze, Germany). Fluconazole (Pfizer UK, Sand-
TABLE 2. Antifungal drug susceptibility of C. glabrata isolates taken from two AIDS patients with OPC

<table>
<thead>
<tr>
<th>Isolate</th>
<th>Fluconazole</th>
<th>Ketokonazole</th>
<th>Itraconazole</th>
<th>Amphotericin B</th>
</tr>
</thead>
<tbody>
<tr>
<td>DSY528</td>
<td>4</td>
<td>0.062</td>
<td>0.125</td>
<td>0.5</td>
</tr>
<tr>
<td>DSY530</td>
<td>32</td>
<td>0.5</td>
<td>1</td>
<td>0.5</td>
</tr>
<tr>
<td>DSY562</td>
<td>4</td>
<td>0.031</td>
<td>0.125</td>
<td>0.5</td>
</tr>
<tr>
<td>DSY565</td>
<td>128</td>
<td>4</td>
<td>4</td>
<td>0.5</td>
</tr>
</tbody>
</table>

Time elapsed between samplings (days)

- DSY528: 20
- DSY530: 47
- DSY562: 20
- DSY565: 50

TABLE 3. Fluconazole accumulation and inhibition of ergosterol biosynthesis by fluconazole in C. glabrata isolates

<table>
<thead>
<tr>
<th>Isolate</th>
<th>Amt of [3H]fluconazole/2 × 10³ cells (cpm)</th>
<th>IC₅₀ of fluconazole for ergosterol biosynthesis (nM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>DSY528</td>
<td>300 ± 20</td>
<td>55 ± 10</td>
</tr>
<tr>
<td>DSY530</td>
<td>95 ± 20</td>
<td>65 ± 8</td>
</tr>
<tr>
<td>DSY562</td>
<td>313 ± 22</td>
<td>40 ± 12</td>
</tr>
<tr>
<td>DSY565</td>
<td>81 ± 5</td>
<td>50 ± 8</td>
</tr>
<tr>
<td>DSY1041</td>
<td>457 ± 7</td>
<td>ND*</td>
</tr>
</tbody>
</table>

* ND, not determined.

FIG. 1. Typing of C. glabrata clinical isolates used in this study. (A) Restriction enzyme analysis of C. glabrata genomic DNA digested with Hinfl. Restriction fragments were separated by 1% agarose gel electrophoresis and stained with ethidium bromide. (B) Profiles of band patterns revealed by hybridization with the repetitive element probes Cg6 and Cg12 as described by Lockhart et al. (29). Molecular size standards were depicted on each photograph.
RESULTS

Origin and azole susceptibility of *C. glabrata* isolates. For the study of mechanisms of resistance to azoles, *C. glabrata* clinical isolates were selected retrospectively from a collection of yeasts recovered from AIDS patients with OPC. The *C. glabrata* strains were chosen from two different patients with recurrent OPC and were first selected on the basis of decreased susceptibility to azole antifungals.

Patient 1 had his first OPC episode in October 1990 and was diagnosed with AIDS. *C. albicans* was isolated initially from the patient’s oral cavity in December 1993 and was subsequently found in recurrent episodes of OPC. In April 1994, the first *C. glabrata* strain (DSY528) was isolated. At that time, patient 1 had received a cumulative dose of 17.3 g of fluconazole, and his CD4 count in blood was 4 cells per mm². After treatment with 400 mg of fluconazole for 7 consecutive days, OPC was still persistent 47 days later. DSY530 was isolated at this time and showed a reduced susceptibility to fluconazole compared to DSY528 (Table 2). Patient 2 had his first clinically documented episode of OPC in November 1991 and was diagnosed with AIDS. *C. albicans* was first isolated from the oral cavity in February 1993. In May 1995, *C. glabrata* DSY562 was isolated for the first time together with *C. albicans*. The patient had received a cumulative dose of 4.1 g of fluconazole, and his CD4 count in blood was 34 cells per mm². After two courses of treatment with 200 mg of fluconazole for 7 consecutive days each, OPC was still persistent 50 days later, and the *C. glabrata* strain DSY565 was isolated, which showed reduced susceptibility to fluconazole compared to that of DSY562 (Table 2).

In both patients, *C. glabrata* isolates were isolated in mixed culture with *C. albicans*. The less susceptible *C. glabrata* isolate of each patient will be designated in this study as azole resistant, without reference to the clinical MIC breakpoints proposed by Rex et al. (44).

The *C. glabrata* strains from these two patients were typed by two different methods. The results shown in Fig. 1 demonstrate that identical banding patterns for the two *C. glabrata* strains from a given patient could be obtained by these methods and thus suggest that the azole-susceptible and azole-resistant isolates from these two patients were related to each other. This implies that no strain replacement occurred during azole therapy in these patients, but rather that azole resistance developed from the original azole-susceptible isolates. The stability of the resistance phenotype did not change after more than 50 consecutive passages (over 500 generations) in drug-free medium. Thus, azole resistance in the azole-resistant isolates could be due to genome alterations rather than to transient adaptation to azole antifungals.

Fluconazole and rhodamine 6G accumulations in *C. glabrata* isolates. We performed several experiments in order to determine the mechanisms of azole resistance in the azole-resistant isolates. First, no changes in amphotericin B susceptibility were observed in the four clinical strains used in this study (Table 2). Therefore, since some alterations in the ergosterol biosynthetic pathway are coupled with amphotericin B resistance (17, 35), it is likely that no alterations in this pathway were occurring in these strains. In agreement with this hypothesis was the detection of this sterol in ergosterol biosynthesis inhibition assays. Second, fluconazole concentrations at which the ergosterol biosynthesis was inhibited in cellular extracts by 50% (IC₅₀) did not exceed the MICs for the tested strains. DNA sequencing. Sequence data were obtained with DNA fragments subcloned from pNB124, pNB125, and pNB126 into pBluescript (Stratagene GmbH, Zürich, Switzerland). Sequence data were generated on both DNA strands by using reverse or universal primers and customized primers by automated sequencing in a Li-Cor 4200 sequencer (Li-Cor, Inc., Lincoln, Nebr.).

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not vary significantly between azole-susceptible and azole-resistant isolates (Table 3). This led us to deduce that an alteration in the affinity of the *C. glabrata* CYP51A1 proteins to azoles was not the cause of azole resistance in these isolates. We therefore tested the accumulation of two different substances in the azole-susceptible and azole-resistant isolates. As shown in Table 3, the azole-resistant isolates were accumulating less fluconazole than their azole-susceptible parents. The accumulation of fluconazole was reduced by factors of 3.8 and 3.1 in DSY565 and DSY530 compared to those in DSY562 and DSY528, respectively. Failure in drug accumulation was not restricted to a single compound, since intracellular levels of rhodamine 6G were also affected in the azole-resistant isolates (Fig. 2). The decreases in rhodamine 6G accumulation were 6.1- and 5.1-fold in DSY565 and DSY530 compared to those in DSY562 and DSY528, respectively. Failure in accumulating several unrelated drugs in the azole-resistant isolates was reminiscent of the effect of multidrug efflux transporters observed in *C. albicans*, and therefore we exploited the possibility that multidrug efflux transporter genes were upregulated in the azole-resistant *C. glabrata* isolates.

**Cloning of *C. glabrata* azole resistance genes.** Given the observations mentioned above, we first attempted to isolate multidrug efflux transporter genes from *C. glabrata*, since at the time these studies were initiated, no *C. glabrata* gene encoding a transporter was available. We used a strategy consisting of isolation of *C. glabrata* genes which could confer azole resistance to an *S. cerevisiae* multidrug efflux transporter mutant. Since the absence of ABC transporter Pdr5p in *S. cerevisiae* yields hypersusceptibility to azole antifungals, this genetic background was suitable to isolate genes involved in azole resistance in *C. glabrata*. From a total of about 80,000 *Ura+* *S. cerevisiae* transformants, more than 200 individual yeasts able to grow on media containing 5 and 10 mg of fluconazole per ml were selected. Each fluconazole-resistant clone was then tested for different drug resistance profiles by using agar plates containing 10 and 25 mg of fluconazole per ml, 0.1 and 1 mg of itraconazole per ml, 0.2 and 2 mg of ketoconazole per ml, 0.25 mg of cycloheximide per ml, 10 mg of fluphenazine per ml, 50 mg of benomyl per ml, 0.5 mg of crystal violet per ml, and 0.5 mg of nitroquinoline-N-oxide per ml. Among the 200 fluconazole-resistant clones, only 20 were specifically resistant to fluconazole among the azole drugs tested while remaining resistant to cycloheximide, benomyl, and nitroquinoline-N-oxide. The other yeast transformants were able to grow on medium containing different azole antifungals and other drugs, such as cycloheximide and fluphenazine. Plasmids rescued from these isolates were extracted and separated by gel electrophoresis.

![Image](https://example.com/image.png)

**FIG. 3.** Cloning of azole resistance genes in *C. glabrata*. (A) Restriction maps of *C. glabrata* insert DNA from plasmids pNB124, pNB125, and pNB126 conferring azole resistance in the *S. cerevisiae* Δpdr5 mutant YKKB-13. The plasmid backbone is YEp24. Sau3A-digested *C. glabrata* DNA was introduced at the BamHI site of YEp24 in the construction of the gene library. The transcription directions of the ORF of each azole resistance gene deduced from nucleotide sequencing are shown by arrows. Ba, BamHI; Bg, BglII; E, EcoRI; Ev, EcoRV; Xba, XbaI; [Ba/Sa], BamHI site of YEp24 destroyed by insertion of a genomic Sau3A site. (B) Drug resistance profiles of *S. cerevisiae* YKKB-13 transformed with plasmids pNB124, pNB125, and pNB126. Yeast strains were spotted in serial dilutions onto YEPD medium containing the drug at the indicated concentration. Plates were incubated at 30°C for 48 h.
To group these plasmids into different categories, C. albicans probes for CDR1, MDR1, and CAP1, which were genes recovered by the same functional complementation (50), were used in low-stringency hybridizations of Southern blots of the C. glabrata plasmids (data not shown). Three different classes of plasmids were recovered, each containing a DNA fragment hybridizing with these probes. From each of these groups, a single plasmid was chosen for further restriction map and sequence analysis. Figure 3A shows the restriction maps of these three plasmids (pNB124, pNB125, and pNB126) with their respective azole resistance genes. pNB124 contained a gene with similarity to C. albicans MDR1 (formerly named BEN), a gene belonging to the class of major facilitator multidrug transporters. This gene was named CgMDR1 (52). pNB125 contained a gene with similarity to the S. cerevisiae YAP1 gene, a transcription factor with a leucine dimerization motif. This gene was therefore named CgYAP1 (52). pNB126 contained a gene with similarity to fungal ABC transporters such as the S. cerevisiae PDR5 gene (8), the C. albicans CDR1 and CDR2 genes (50, 53) or the recently cloned CgYAP1 gene (32). This C. glabrata gene was named CgCDR1 for Candida drug resistance gene, by analogy with the C. albicans CDR genes. Figure 3B shows the drug resistance profiles obtained because of the presence of each plasmid in YKKB-13. All plasmids could confer resistance to fluconazole, but only pNB126 containing CgCDR1 conferred resistance to differentazole antifungals and fluphenazine. pNB124 and pNB125 conferred similar drug resistance profiles, but only pNB124 containing CgMDR1 was able to confer slight resistance to benomyl.

Expression of azole resistance genes in C. glabrata. We next performed Northern blot analysis of total RNA extracted from C. glabrata clinical isolates DSY562, DSY565, DSY528, and DSY530 with DNA probes for each of the azole resistance genes. This experiment was aimed to show a possible correlation between the expression of an azole resistance gene and azole resistance in the clinical isolates. The CgURA3 gene was used as an internal control for the standardization of signals. The CgERG11 gene was also included as a probe in this analysis, since it was reported by others that CgERG11 overexpression can moderately establish azole resistance in C. glabrata (31). Figure 4A shows that only CgCDR1 expression was elevated in both azole-resistant isolates from the two different patients. The relative increases in CgCDR1 expression observed in DSY565 and DSY530 were eight- and fivefold compared to those in DSY562 and DSY528, respectively (Fig. 4B). The expression of the other genes was not significantly affected compared to those in DSY562 and DSY528, respectively (Fig. 4B). Figure 5 shows the nucleotide sequence of a 5.3-kb fragment isolated from pNB126 starting from the Sau3A cloning site on YEp24 and ending at the single BamHI site of the same plasmid. An uninterrupted ORF of 4,500 bp encoding a protein with 1,499 amino acids was detected from the most upstream ATG codon. The 5′ region starting from this ATG codon displayed typical features of yeast promoters: an adenine at position −3 and a TATA box at position −83 could be distinguished (Fig. 5). We noticed the presence of four putative Pdr1p and Pdr3p binding sites in the 5′ flanking region of CgCDR1. The positions of these sites were as follows:

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FIG. 4. Expression of azole resistance genes in C. glabrata clinical isolates. (A) Northern blot analysis. Five micrograms of RNA from each strain was separated and blotted as indicated in Materials and Methods. The blotted membrane was probed sequentially with 32P-labelled probes for CgCDR1, CgYAP1, CgMDR1, CgERG11, and CgURA3. Labelled DNA probes are described in Materials and Methods. Washed membranes were exposed to Fujix RX films for 1 to 10 h. (B) Quantification of hybridization signals detected by each 32P-labelled probe. Signals given by each probe were counted with an Instant Imager and normalized to the counts obtained with the CgURA3 probe. The values of the normalized counts for CgMDR1 were too small to be reported in the figure. These values were 0.0093 for DSY528 and 0.012 for DSY530, and 0.037 for DSY562 and 0.026 for DSY565.

FIG. 5. Nucleotide and deduced amino acid sequences of the CgCDR1 gene and its encoded polypeptide. Nucleotides and amino acids are numbered to the left and to the right, respectively. The sequence starts from a Sau3A site and ends with a BamHI site. The CgCDR1 ORF is indicated below the nucleotide sequence, and its position in the pBN126 restriction map corresponds to the arrow shown in Fig. 3A. In the deduced protein sequence, putative membrane-spanning domains are underlined and the Walker A and B ATP-binding motifs and ABC signatures are boxed in the N- and C-terminal regions. A TATA box in the 5′ flanking region is underlined. Putative Pdr1p and Pdr3p binding sites in the 5′ flanking region are boxed. The CgCDR1 sequence is available in the GenBank database under accession no. AF109723.
AZOLE RESISTANCE IN C. GLABRATA

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The consensus of the Pdr1p and Pdr3p binding sites in the S. cerevisiae PDR5 gene was 7TCCG(C/T)GGAA. Nucleotides conserved among the Pdr1p and Pdr3p binding sites are shown in boldface letters. Palindromes are underlined. These sites (and conserved nucleotide sequences) have been shown to be important in the regulation of ABC transporter genes in S. cerevisiae by the transcription factors Pdr1p and Pdr3p (23). These sites may therefore play a similar role in the regulation of CgCDR1.

The CgCDR1 ORF encodes a protein of 169 kDa (CgCdr1p) and displays a structure and domain organization typical of membrane proteins of the ABC superfamily (Fig. 5). A blast search of the CgCDR1 ORF against the entire GenBank database gave the highest score with PDR5 from S. cerevisiae. C. glabrata CgCdr1p is 75% identical to Pdr5p. CgCdr1p is composed of two homologue halves, each comprising a C-hydrophilic domain and a N-hydrophobic domain. The hydrophathy plot of CgCdr1p identified six putative transmembrane domains for each of the hydrophobic domains (Fig. 5). Each hydrophilic domain included ATP-binding motifs found in ATP-binding cassette domains. In the N-terminal ABC domain, the Walker A (LGRPGSGCTTLL) and B (QCWDNAATLGD) motifs and the ABC signature (SGGERKRVSIAE) could be recognized. In the C-terminal ABC domain, both the Walker A (GASAGKTTL) and Walker B (VFLEPSGLD) regions are present, but an atypical ABC signature (NVQORKRLTIGV) is present where the usual SGX/G/E is replaced by NVEQ. Recently Miyazaki et al. (32) reported the nucleotide sequence of an ABC transporter gene from C. glabrata called Pdh1p, however, was not identical to CgCdr1p, as revealed by pairwise alignment (data not shown). Both proteins showed 75% identity, which was most prominent in the ABC signatures and Walker A and B domains.

**Construction of CgCDR1 mutants in C. glabrata.** Given the overexpression of CgCDR1 in the azole-resistant clinical isolates, we addressed the involvement of this ABC transporter inazole resistance by the deletion of this gene in one azole-resistant isolate. First, an auxotrophic mutant for uracil was generated by positive selection of resistance to 5-FOA. 5-FOA-resistant colonies from DSY565 were obtained with a low reversion frequency (10−5). The identity of the ura3 auxotrophy could be confirmed, since, by transformation of one 5-FOA-resistant DSY565 derivative (DSY1029) with a C. glabrata plasmid containing the CgURA3 gene, Ura+ colonies were obtained (data not shown). DSY1029 was further utilized for the deletion of CgCDR1. A linear fragment which contained the hisG-URA3-hisG cassette from pNK51 (2) replacing an internal deletion of CgCDR1 (Fig. 6A) was used to transform DSY1029. Ura+ colonies were selected, and analysis of the CgCDR1 locus in one of the Ura+ transformants (DSY1041) confirmed that the correct gene replacement had occurred (Fig. 6B). The wild-type CgCDR1 6-kb EcoRI fragment observed in both DSY562 and DSY565 was not observed in DSY1041 and was replaced by a 9.4-kb band expected from the insertion of the hisG-URA3-hisG cassette. To verify if the deletion made in CgCDR1 affected its expression, total RNA from DSY1041 was analyzed by Northern analysis with a CgCDR1 probe. As shown in Fig. 7, a weak signal could still be detected in RNA from DSY1041, but with a slightly reduced migration when compared to the CgCDR1 mRNA signal observed in DSY562 and DSY565. The intensity of this signal was, however, much reduced compared to that of the CgCDR1 mRNA signal in DSY565. Therefore, deletion of CgCDR1 in DSY565 affected the expression of this gene to a large extent. The weak signal still observed in DSY1041 could be attributed to the formation of aberrant CgCDR1 mRNA or to the presence of additional mRNA(s) from an additional ABC transporter gene or genes hybridizing weakly with the CgCDR1 probe.

DSY1041 was subjected to azole susceptibility tests and drug accumulation experiments in parallel to DSY562 and DSY565. As shown in Fig. 8, the deletion of CgCDR1 in DSY1041 had a significant effect on the susceptibility to azoles. DSY1041 could not grow at drug concentrations permissive for DSY565 in medium containing fluconazole, itraconazole, or ketocon-
azole. DSY1041 was also more susceptible to azole antifungal agents in this assay than the azole-susceptible isolate DSY562, as revealed by the difference in growth between both yeasts in the azole-containing YEPD medium tested. Since $CgCDR1$ was still expressed in DSY562 as opposed to DSY1041, this result could, however, be expected. MICs of azole antifungals for DSY1041 measured with the NCCLS standard protocol in RPMI medium were lower than those for DSY565 and were in the range of those measured for DSY562 (Table 4). We noticed that the MICs of fluconazole were lower for DSY1041 than for DSY562, when other incubation media were used, in particular YNB (data not shown). Intracellular levels of fluconazole and rhodamine 6G were restored in DSY1041 compared to those in DSY565 and reached or exceeded the levels found in DSY562. As shown in Table 3, fluconazole accumulation was increased by 5.6-fold in DSY1041 compared to that in DSY565. The accumulation of rhodamine 6G in DSY1041 was increased by a factor of 16.5 compared to that in DSY565 (Fig. 9). We also observed that the efflux of rhodamine 6G in DSY1041 loaded with this substance was much reduced compared to that in DSY565 or DSY562 (data not shown). Taken together, these results showed that the deletion of $CgCDR1$ in theazole-resistant isolate DSY565 dramatically affected azole resistance and drug accumulation and thus support the idea that the expression of $CgCDR1$ was the major cause of azole resistance in this isolate.

Rescue of fluconazole resistance phenotype by $CgCDR1$. In order to identify the nature of the mutation or mutations responsible for the increase in $CgCDR1$ mRNA in the azole-resistant clinical isolate DSY565, $CgCDR1$ isolated from an azole-susceptible $C. glabrata$ strain was reintroduced in the $CgCDR1$ deletion mutant DSY1041 (the $ura3$ derivative of DSY1041) with the help of a replicating vector. Since 2μm-derived vectors do not efficiently replicate in $C. glabrata$, pNB126, which contains $CgCDR1$ on a 2μm-based vector, was equipped with the $CgCEN$ and $CgARS$ centromeric elements from $C. glabrata$, thus enabling the replication of the resulting construction (pDS670) in a single copy in $C. glabrata$ (25). As shown in Fig. 10A, the presence of pDS670 in DSY1053 (DSY1717) restored the azole resistance phenotype. This phenotype was followed by a 6.5-fold increase in $CgCDR1$ mRNA in DSY1717 (Fig. 10B). The same plasmid, pDS670, was used to transform DSY1067, which is the $ura3$ derivative of DSY1033, a $CgCDR1$ deletion mutant derived from the azole-susceptible laboratory strain DSY671 (61). The resulting strain, DSY1718, was as susceptible as DSY671 (Fig. 10A). $CgCDR1$ mRNA levels in DSY1718 were 1.6-fold higher than those in DSY671 and thus were comparable (Fig. 10B). Taken together, these results suggest that the higher $CgCDR1$ expression levels in DSY1717 should be the consequence of a trans-acting mutation(s) rather than of a cis-acting mutation or mutations on $CgCDR1$. cis-acting mutations on $CgCDR1$ from DSY565 would have been revealed if pDS670 had produced similar azole susceptibility and similar $CgCDR1$ mRNA levels in both DSY1717 and DSY1718. The results obtained in Fig. 10 reflect the pleiotropic drug resistance phenomenon in S. cerevisiae, which is controlled by several mutations of transcription factors acting in trans on several ABC transporter genes (9).

**DISCUSSION**

OPC represents one of the main mucosal infections in AIDS patients. While $C. albicans$ remains the principal agent of OPC, $C. glabrata$ is often recovered from clinical samples in addition to $C. albicans$. Among 298 human immunodeficiency virus-positive patients monitored in our institution for the occurr-

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**TABLE 4. Azole susceptibility of $C. glabrata$ clinical isolates and of the ABC transporter mutant DSY1041**

<table>
<thead>
<tr>
<th>Isolate</th>
<th>MIC (μg/ml)</th>
<th>Fluconazole</th>
<th>Ketoconazole</th>
<th>Itraconazole</th>
<th>Amphotericin B</th>
</tr>
</thead>
<tbody>
<tr>
<td>DSY562</td>
<td>4</td>
<td>0.062</td>
<td>0.25</td>
<td>0.5</td>
<td></td>
</tr>
<tr>
<td>DSY565</td>
<td>128</td>
<td>2</td>
<td>4</td>
<td>0.5</td>
<td></td>
</tr>
<tr>
<td>DSY1041</td>
<td>8</td>
<td>0.25</td>
<td>0.5</td>
<td>0.5</td>
<td></td>
</tr>
</tbody>
</table>
We describe here a mechanism of resistance to azole antifungals in two distinct azole-resistant C. glabrata isolates from two different AIDS patients with OPC. The C. glabrata strains used in this study were chosen on the basis of two criteria. First, two pairs of isolates in which changes in azole susceptibility were measured during fluconazole therapy were selected. Second, the isolates of each pair were compared by two typing methods to verify their relatedness. The isolates of the present study satisfied these criteria. We observed retrospectively that the times that elapsed between the recovery of the first azole-susceptible C. glabrata isolate and the azole-resistant isolate were not only short (47 versus 50 days) but were also similar in the two patients. Miyazaki et al. (32) in their investigations of azole-resistant isolates from 0.5% in 1990 to 22.2% in 1996, mainly originating from high-risk patients in the intensive care unit and the oncology ward. A recent study retrospectively reviewing medical records of patients with hematogenous candidiasis revealed a relative decrease in C. albicans and C. tropicalis infections, but an increase in C. krusei and C. glabrata infections (1). Fluconazole prophylaxis was the single most important determinant for the relative increase in C. krusei and C. glabrata infections. Similar shifts in yeast population favoring Candida spp. are also reported by others (22, 26). These trends suggest that C. glabrata is becoming increasingly important, and due to the intrinsic low susceptibility of this yeast to azole antifungals, the management of fungal diseases by azole antifungals is rendered more difficult.

We describe here a mechanism of resistance to azole antifungals in two distinct azole-resistant C. glabrata isolates from two different AIDS patients with OPC. The C. glabrata strains used in this study were chosen on the basis of two criteria. First, two pairs of isolates in which changes in azole susceptibility were measured during fluconazole therapy were selected. Second, the isolates of each pair were compared by two typing methods to verify their relatedness. The isolates of the present study satisfied these criteria. We observed retrospectively that the times that elapsed between the recovery of the first azole-susceptible C. glabrata isolate and the azole-resistant isolate were not only short (47 versus 50 days) but were also similar in the two patients. Miyazaki et al. (32) in their investigations of azole resistance in C. glabrata reported an elapsed time of 2 weeks between the sampling of azole-susceptible and the azole-resistant isolates in one AIDS patient under fluconazole therapy. This feature has been also noticed in our collection of C. glabrata isolates and suggests rapid changes in azole susceptibility in C. glabrata strains exposed to azole antifungals. This observation needs to be confirmed by other laboratories.

The preliminary experiments which helped us to determine the cause of resistance in the azole-resistant C. glabrata isolates excluded changes of affinity to azoles in CYP51A1 and also eliminated alteration of sterol biosynthesis, since no differences in amphotericin B susceptibility between azole-susceptible and azole-resistant isolates were measured. Failure of drug accumulation in the azole-resistant isolates was the remaining known mechanism and proved to be present in both azole-resistant isolates. Drug accumulation studies were performed with two different substances, [3H]fluconazole and rhodamine 6G. Rhodamine 6G is a substrate for several ABC transporters and has been used for efflux studies with intact S. cerevisiae cells through the convenient method of flow cytometry (12, 13). The use of rhodamine 6G for measuring accumulation differences by FACS analysis in the C. glabrata isolates of this study has proved to be a very simple method which is much less time-consuming than the method utilizing 3H-labelled fluconazole. By the use of both substrates, not only could the cause of reduced azole susceptibility in isolates DSY530 and DSY565 be explained as a drug accumulation failure, but the nature of this process as an effect due to multidrug transport could be determined as well. FACS analysis of rhodamine 6G-labelled yeast cells can represent a rapid method for the study of azole resistance in a large collection of clinical isolates; however, this method first needs to be validated with more azole-resistant isolates.

The functional cloning of azole resistance genes from C. glabrata greatly assisted the identification of genes involved in azole resistance in the clinical isolates. This strategy had been used already for the cloning of similar genes in C. albicans and revealed six genes, among which CDR1, CDR2, and C. albicans MDRI were shown to be upregulated in azole-resistant strains (50, 53). In this study, we could identify three different C. glabrata azole resistance genes: a transcription factor (CgYAP1), a multidrug transporter of the major facilitator family (CgMDRI), and a multidrug transporter of the ABC transporter family (CgCDRI). Only the transcription of CgCDRI was increased in the azole-resistant isolates examined in this study. This effect has also been observed in at least 15 other azole-resistant C. glabrata isolates from AIDS patients of our institution (47), thus suggesting that this mechanism of resistance is common in C. glabrata. Reports on the characterization of azole resistance in C. glabrata from other laboratories have pointed to a failure of drug accumulation as a cause of resistance. Hitchcock et al. (20), Parkinson et al. (38), and Miyazaki et al. (32) reported differences of fluconazole accumulation in azole-resistant C. glabrata isolates from other laboratories with altered sterol biosynthesis pathway have not yet been detected in C. glabrata clinical strains. However, laboratory strains with altered sterol profiles have been constructed by deletion of the ERG3 and ERG11 genes (17). Only the simultaneous deletion of both genes yielded mutants with azole and amphotericin B resis-
tance, the latter being consistent with the absence of ergosterol in these cells. Thus, the pathway of azole resistance involving ergosterol biosynthesis may be utilized in clinical strains, but requires multiple gene alterations.

As mentioned above, another ABC transporter gene from *C. glabrata* called *PDH1* has recently been isolated (32). *PDH1*, which is distinct from *CgCDR1*, was cloned by using PCR primers matching a consensus in conserved elements among ABC transporters and not by functional complementation as performed in this study. The function of *PDH1* as a multidrug transporter has not yet been demonstrated, as opposed to *CgCDR1*, which is able to confer resistance to multiple drugs when expressed in *S. cerevisiae* (Fig. 3B). In the report of Miyazaki et al. (32), *PDH1* expression was increased between azole-susceptible and azole-resistant *C. glabrata* isolates, as is the case here for *CgCDR1*. What is the relevance of both ABC transporter genes for the development of azole resistance in clinical *C. glabrata* isolates? In the light of our results, since the deletion of *CgCDR1* in DSY565 rendered the mutant strain as susceptible to azoles as the susceptible parent isolate (DSY562), it indicates that *PDH1* should play a moderate role in the azole resistance of this isolate. This hypothesis is further validated by Northern blot analysis, in which *PDH1* mRNA signals were absent in total RNA from DSY562 while present in DSY565, but with a much reduced intensity compared to *CgCDR1* mRNA signals (46). *PDH1* may, however, be expressed at higher levels in other not-yet-investigated *C. glabrata* azole-resistant strains, a possibility which remains to be tested. The presence of multiple ABC transporter genes being coordinately upregulated is a feature known in other azole-resistant yeasts, such as *C. albicans* (50). *CDR1* and *CDR2*, which are two ABC transporter genes with high similarity, are upregulated in azole-resistant isolates, while only *CDR1* expression is detected in azole-susceptible strains, thus paralleling the properties of *CgCDR1* and *PDH1*.

The rescue of the azole resistance phenotype by *CgCDR1* in the ABC transporter mutant DSY1041 (Fig. 10) is an important result which will aid the further molecular characterization of the azole resistance mechanism in *C. glabrata*. By identifying *trans*-acting factors rather than *cis*-acting factors implicated in the upregulation of *CgCDR1* in the azole-resistant isolate DSY565, the role of transcription factors regulating the expression of *CgCDR1* becomes more relevant. The presence of putative Pdr1p and Pdr3p binding sites in the promoter region of *CgCDR1* suggests strongly that these types of transcription factors might operate in *C. glabrata*. Interestingly, these binding sites have also been recognized in the promoter region of *PDH1* (32). The function of Pdr1p and Pdr3p in *S. cerevisiae* has been well investigated for the regulation of the ABC transporter gene *PDR5*. The absence of Pdr1p and Pdr3p binding sites in the promoter of *PDR5* decreases the expression of this gene to almost undetectable levels, but increases the expression of *CgCDR1* when expressed in *S. cerevisiae*. This is consistent with the idea that *PDH1* expression plays a limited role in the azole resistance of *C. glabrata*, while *CgCDR1* may have a more dominant role.
levels (23). PDR1 and PDR3 gain-of-function alleles have been reported in S. cerevisiae to confer hypersensitivity to multiple drugs by increasing the transcription of several target genes, among which is the ABC transporter gene PDR5 (9, 36). A similar situation may occur in C. glabrata. PDR1 and PDR3 homologues are likely to be present in C. glabrata, and gain-of-function alleles of these genes could activate in trans the expression of CcCdr1 or Pdh1 in azole-resistant strains.

In summary, this study demonstrates for the first time that azole resistance in clinical C. glabrata isolates is mediated by the upregulation of an ABC transporter gene. It is likely that not-yet-characterized multidrug efflux transporters may participate in this process, a hypothesis currently being addressed in our laboratory. A study of the molecular mechanisms of resistance to azoles in C. glabrata is attractive not only because this yeast has become an important human pathogen in recent years but also because recently developed genetic tools allow the testing of different working hypotheses.

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REFERENCES


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46. Sanglard, D. Unpublished data.
47. Sanglard, D., and D. Calabrese. Unpublished data.