Mechanisms of Azole Resistance in Clinical Isolates of *Candida glabrata* Collected during a Hospital Survey of Antifungal Resistance

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The increasing use of azole antifungals for the treatment of mucosal and systemic *Candida glabrata* infections has resulted in the selection and/or emergence of resistant strains. The main mechanisms of azole resistance include alterations in the *C. glabrata* *ERG11* gene (*CgERG11*), which encodes the azole target enzyme, and upregulation of the *CgCDR1* and *CgCDR2* genes, which encode efflux pumps. In the present study, we evaluated these molecular mechanisms in 29 unmatched clinical isolates of *C. glabrata*, of which 20 isolates were resistant and 9 were susceptible dose dependent (S-DD) to fluconazole. These isolates were recovered from separate patients during a 3-year hospital survey for antifungal resistance. Four of the 20 fluconazole-resistant isolates were analyzed together with matched susceptible isolates previously taken from the same patients. Twenty other azole-susceptible clinical *C. glabrata* isolates were included as controls. MIC data for all the fluconazole-resistant isolates revealed extensive cross-resistance to the other azoles tested, i.e., itraconazole, ketoconazole, and voriconazole. Quantitative real-time PCR analyses showed that *CgCDR1* and *CgCDR2*, alone or in combination, were upregulated at high levels in all but two fluconazole-resistant isolates and, to a lesser extent, in the fluconazole-S-DD isolates. In addition, slight increases in the relative level of expression of *CgSNQ2* (which encodes an ATP-binding cassette [ABC] transporter and which has not yet been shown to be associated with azole resistance) were seen in some of the 29 isolated strains. Interestingly, the two fluconazole-resistant isolates expressing normal levels of *CgCDR1* and *CgCDR2* exhibited increased levels of expression of *CgSNQ2*. Conversely, sequencing of *CgERG11* and analysis of its expression showed no mutation or upregulation in any *C. glabrata* isolate, suggesting that *CgERG11* is not involved in azole resistance. When the isolates were grown in the presence of fluconazole, the profiles of expression of all genes, including *CgERG11*, were not changed or were only minimally changed in the resistant isolates, whereas marked increases in the levels of gene expression, particularly for *CgCDR1* and *CgCDR2*, were observed in either the fluconazole-susceptible or the fluconazole-S-DD isolates. Finally, known ABC transporter inhibitors, such as FK506, were able to reverse the azole resistance of all the isolates. Together, these results provide evidence that the upregulation of the *CgCDR1*, *CgCDR2*, and *CgSNQ2*-encoded efflux pumps might explain the azole resistance in our set of isolates.

*Candida glabrata* has recently emerged as a significant pathogen in various hospital settings, where it is responsible for an increasing number of systemic infections and candiduriasis (2, 16). In a recent study, *C. glabrata* was the second most common non-*C. albicans* species as a cause of fungemia in the United States and was found to account for 21% of all *Candida* bloodstream isolates (26). Second only to *C. albicans*, *C. glabrata* is also the *Candida* species most commonly recovered from the oral cavities of human immunodeficiency virus-infected patients (13, 16, 40).

The rise in the number of *C. glabrata* systemic infections deserves a great deal of concern due to the high mortality rate associated with *C. glabrata* fungemia and to the propensity of this microorganism to rapidly develop resistance to azole antifungal agents (10, 19). Several studies have revealed that a significant percentage of *C. glabrata* clinical isolates are resistant to fluconazole (approximately 9%) and itraconazole (37 to 40%) (3, 16, 25). More recently, in a surveillance study conducted by Pfaffer et al. (27) to examine the antifungal susceptibilities of *Candida* species isolated from patients with bloodstream infections stratified by patient age, a trend of decreasing susceptibilities to fluconazole and itraconazole with increasing patient age was observed. In fact, none of the *C. glabrata* isolates from individuals ≤1 year old were resistant to fluconazole, whereas a higher proportion (5 to 9%) of resistant isolates was found in adult patients. Similarly, among 347 bloodstream, invasive, and colonizing strains of *C. glabrata* isolated from patients at three urban teaching hospitals in New York City, the overall rates of resistance to fluconazole and itraconazole were 10.7 and 15.2%, respectively (33).

The mechanisms of resistance to azole antifungal agents have been well elucidated in *C. albicans* and can be mainly categorized as (i) changes in the cell wall or plasma membrane, which lead to impaired azole uptake; (ii) alterations in the affinity of the drug target Erg11p (14α-demethylase) to azoles or in the cellular content of Erg11p due to target site mutation or overexpression of the *ERG11* gene; and (iii) the efflux of drugs mediated by membrane transport proteins belonging to the ATP-binding cassette (ABC) transporter family (*CDR1* and *CDR2*) or to the major facilitator superfamily...
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(MDR1 and FLU1). In the last case, the CDR1 and CDR2 genes and the MDR1 gene were shown to be overexpressed in many resistant isolates, and deletion of these genes resulted in hypersensitivity to azoles (34). In addition, compensatory pathways that involve alterations of specific steps in ergosterol biosynthesis have been documented as mechanisms of resistance to the azole and polyene antifungal classes (39).

More recently, increased levels of expression of the ABC transporter genes C. glabrata CDR1 (CgCDR1) and CgCDR2 have been shown to be azole-resistant isolates of C. glabrata (5, 15, 35, 36). Similar to C. albicans, genetic evidence supporting the role of multidrug transporters in theazole resistance of C. glabrata was provided (36). Moreover, Mariscal et al. (14) previously showed increased levels of expression of ERG11 in an azole-resistant C. glabrata strain which arose from a chromosomal duplication. In contrast, it has yet to be well explored whether point mutations in the ERG11 gene are also implicated in the resistance of C. glabrata to azoles.

The purpose of the present study was to determine if the molecular mechanisms described above, alone or in combination, were sufficient to explain the phenotype of azole resistance in unmatched clinical C. glabrata isolates obtained from various clinical specimens during a 3-year hospital survey of antifungal resistance or if other (not well-established) mechanisms might correlate with azole resistance. In addition, pairs of susceptible and resistant C. glabrata isolates that had been obtained from the same patient and that had the same genotype were also examined.

MATERIALS AND METHODS

Yeast isolates and growth conditions. The isolates of C. glabrata included in the present study were from a collection of clinical isolates recovered during an epidemiological survey of antifungal resistance conducted at our institution, a large university hospital in Rome, over a 3-year period (January 2000 through December 2003). They were identified by standard methods (43) and tested for their susceptibilities to amphotericin B, fluconazole, fluconazole, ketoconazole, itraconazole, and voriconazole by the NCCLS reference method for confirmation and were then subjected to the molecular analysis already described (21). A twofold increase in the level of expression of each gene was considered significant.

Cloning and sequencing of ERG11 gene. For amplification of the ERG11 genes from the C. glabrata isolates, primers ERG11fw (5'-ATGTCACCTGAACACTTCTTGG-3') and ERG11rev (5'-GATCTTGTGATGATGTCCTTTTC-3') were designed on the basis of positions 561 to 2159 of the CgERG11 sequence (GenBank accession no. LF40389). PCRs were carried out with a 50-μl volume containing 1X Pfu amplification buffer (Invitrogen); dATP, dGTP, dCTP, and dTTP (Roche Diagnostics, Milan, Italy) at a concentration of 200 μM each; primers ERG11a and ERG11b (synthesized by MWG Biotech) at a concentration of 5 μM each; 1 U of Platinum Pfu DNA polymerase (Invitrogen); and 100 ng of genomic DNA. Amplification conditions were as follows: 95°C for 2 min, 95°C for 15 s, 57°C for 1 min, and 68°C for 2 min repeated for 40 cycles. The PCR products were purified and cloned into the Zero Blunt vector system (Invitrogen). The insert DNAs of the recombinant plasmids were sequenced automatically with an ABI Prism 377 sequencer analyzer (Applied Biosystems, Foster City, Calif.). For each clone, the sequences of both strands were analyzed, and multiple-amino-acid alignments were derived by using WinDNASYS software (Hitachi Software Genetic Systems, San Francisco, Calif.).

Exposure of yeast isolates to fluconazole. For fluconazole exposure, we followed the protocol described by Niimi et al. (20). Susensions of C. glabrata cells (OD650 0.1) freshly prepared in YEPD medium were grown at 30°C to reach an OD650 of 0.3; then, fluconazole was added at a final concentration of 100 μg/ml and the cultures were incubated at 30°C for a further 4 h. Preliminary experi-
ments showed that 4 h was the optimal time for induction (data not shown). In addition, resistant isolates were also exposed to higher concentrations of fluconazole for 10 min and washed twice with sterile water, and the DNA was extracted by liquid lysate with the QIAgen kit in accordance with NCCLS document M27-A2 (17).

**TABLE 1.** Sites of isolation, antifungal susceptibilities, and resistance mechanisms for the 33 *C. glabrata* isolates studied

| Isolate designation | Site of isolation | MIC (µg/ml) | FLC susceptibility category | Gene(s) overexpressed (fold increase) |
|---------------------|------------------|-------------|----------------------------|-------------------------------------|
|                     |                  | FLC | ITC | KTC | VOR |               |                        |
| BPY40               | Blood            | 4   | 0.25 | 0.25 | 0.125 | S                 |                       |
| BPY41               | Blood            | 256 | >16  | 4    | 8     | R   | CgCDR1 (483.1), CgCDR2 (70.3), CgSNQ2 (11.7) |
| BPY112              | Blood            | 2   | 0.125 | 0.125 | 0.125 | S                 |                       |
| BPY126              | Blood            | 128 | 16   | 4    | 2     | R   | CgCDR1 (56.2), CgCDR2 (5.5), CgSNQ2 (18.7) |
| BPY241              | Blood            | 4   | 0.25 | 0.125 | 0.125 | S                 |                       |
| BPY285              | Blood            | 128 | 2    | 2    | 4     | R   | CgCDR1 (51.0), CgCDR2 (13.3) |
| BPY449              | Blood            | 4   | 0.25 | 0.25 | 0.125 | S                 |                       |
| BPY479              | Blood            | 256 | 2    | 4    | 2     | R   | CgCDR1 (132.8), CgCDR2 (45.5) |

Unmatched isolates

| Isolate designation | Site of isolation | MIC (µg/ml) | FLC susceptibility category | Gene(s) overexpressed (fold increase) |
|---------------------|------------------|-------------|----------------------------|-------------------------------------|
|                     |                  | FLC | ITC | KTC | VOR |               |                        |
| BPY42               | BAL<sup>d</sup> fluid | 128 | >16 | 4    | 4    | R   | CgCDR1 (31.9) |
| BPY43               | Urine/catheter   | 128 | >16 | 4    | 4    | R   | CgCDR1 (100.4) |
| BPY44               | Vagina           | 128 | >16 | 4    | 4    | R   | CgCDR1 (61.3) |
| BPY45               | Blood            | 256 | >16 | 4    | 4    | R   | CgCDR1 (130.4) |
| BPY46               | Blood            | 128 | >16 | 4    | 4    | R   | CgCDR1 (141.0), CgCDR2 (28.4), CgSNQ2 (5.1) |
| BPY47               | Urine            | 128 | >16 | 4    | 4    | R   | CgCDR1 (90.0), CgCDR2 (6.8) |
| BPY48               | Vagina           | 128 | >16 | 2    | 2    | R   | CgSNQ2 (25.3) |
| BPY49               | Urine/catheter   | 256 | >16 | 4    | 4    | R   | CgCDR1 (263.9), CgCDR2 (3.8), CgSNQ2 (5.5) |
| BPY50               | Oral cavity      | 256 | 16   | 8    | 4    | R   | CgCDR1 (227.1), CgCDR2 (34.7) |
| BPY54               | Sputum           | 128 | 16   | 2    | 2    | R   | CgCDR1 (18.0), CgSNQ2 (3.7) |
| BPY55               | Vagina           | 64  | 4    | 1    | 2    | R   | CgSNQ2 (14.7) |
| BPY57               | Urine            | 64  | 2    | 1    | 16   | R   | CgCDR1 (11.9) |
| BPY59               | Oral cavity      | 64  | 2    | 1    | 1    | R   | CgCDR2 (31.7), CgSNQ2 (12.7) |
| BPY60               | Decubitus ulcer  | 256 | 16   | 4    | 8    | R   | CgCDR1 (235.5), CgCDR2 (34.0), CgSNQ2 (20.5) |
| BPY135              | Urine/catheter   | 64  | 2    | 1    | 1    | R   | CgCDR2 (25.9), CgSNQ2 (9.9) |
| BPY233              | Drainage fluid   | 256 | 16   | 4    | 4    | R   | CgCDR1 (79.1), CgCDR2 (13.3) |
| BPY51               | Vagina           | 16  | 1    | 0.5  | 0.25 | S-DD | CgCDR1 (5.6), CgSNQ2 (2.9) |
| BPY52               | Urine/catheter   | 32  | 0.5  | 0.5  | 0.25 | S-DD | CgCDR1 (8.0), CgSNQ2 (4.7) |
| BPY53               | Blood            | 16  | 0.5  | 0.5  | 0.25 | S-DD | CgCDR1 (3.8), CgCDR2 (4.8) |
| BPY56               | Sputum           | 16  | 0.125 | 0.06 | 0.125 | S-DD | CgCDR1 (4.4) |
| BPY58               | Urine/catheter   | 16  | 1    | 0.5  | 1    | S-DD | CgCDR2 (4.7), CgSNQ2 (6.1) |
| BPY150              | Urine/catheter   | 32  | 2    | 0.5  | 0.5  | S-DD | CgCDR1 (3.7) |
| BPY174              | Urine/catheter   | 16  | 2    | 0.25 | 0.25 | S-DD | CgCDR1 (5.4) |
| BPY221              | Abscess fluid    | 16  | 1    | 0.125 | 0.25 | S-DD | CgCDR1 (6.5) |
| BPY270              | Feces<sup>e</sup> | 16  | 1    | 0.25 | 0.25 | S-DD | CgCDR1 (6.0) |

<sup>a</sup> FLC, fluconazole; ITC, itraconazole; KTC, ketoconazole; VOR, voriconazole. The MICs of these antifungal agents were determined by the broth microdilution method, in accordance with NCCLS document M27-A2 (17).

<sup>b</sup> S, susceptible (MIC, ≥8 µg/ml); R, resistant (MIC, ≥64 µg/ml).

<sup>c</sup> Quantification was performed by real-time RT-PCR (see the text for details). The values are averages of four independent experiments and represent increases in the levels of gene expression relative to the levels measured in the absence of fluconazole.

<sup>d</sup> BAL, bronchoalveolar lavage.

<sup>e</sup> Stool specimen from a patient with an absence of normal bacterial flora.

**RESULTS**

Antifungal susceptibilities of *C. glabrata* isolates. The *C. glabrata* isolates studied here were obtained during a hospital course of therapy.

*Statistical analysis.* The two-tailed Student’s *t* test was used to compare categorical variables. A *P* value of less than 0.05 was considered statistically significant.
survey conducted at our institution to monitor the antifungal resistance patterns of *Candida* sp. infections over a 3-year period. A total of 267 *C. glabrata* isolates from consecutive infections were screened for antifungal resistance by a routine susceptibility testing method in use in our laboratory and adapted from the NCCLS reference method (8, 24). Figure 1 shows the rates of azole resistance for these *C. glabrata* isolates. Among the resistant isolates, 7.5, 9.7, 7.5, and 6.7% were resistant to fluconazole, itraconazole, ketoconazole, and voriconazole, respectively, whereas all isolates were found to be susceptible to amphotericin B and fluocytosine. The fluconazole MICs for the 29 *C. glabrata* isolates that originated from different patients hospitalized in intensive care units and oncology wards were superior to the susceptibility breakpoint established for this agent (MIC, \( \leq 8 \) μg/ml), and the isolates were selected for further phenotypic characterization. These isolates were tested for their susceptibilities to a panel of four azole antifungal agents, including fluconazole, itraconazole, keto-

| Isolate designation | Site of isolation | MIC (μg/ml)* | Gene expression (fold increase)a | CgCDR1 | CgCDR2 | CgSNQ2 | ERG11 |
|---------------------|------------------|--------------|---------------------------------|--------|--------|--------|--------|
| BPY104              | Vagina           | 4            | 0.25                             | 0.125  | 0.25   | 0.125  | 0.125  |
| BPY140              | Sputum           | 2            | 0.06                             | 0.06   | 0.06   | 0.06   | 0.06   |
| BPY164              | Blood            | 2            | 0.125                            | 0.125  | 0.125  | 0.125  | 0.125  |
| BPY197              | Oral cavity      | 4            | 0.5                              | 0.125  | 0.125  | 0.125  | 0.125  |
| BPY222              | Urine/catheter   | 4            | 0.25                             | 0.125  | 0.125  | 0.125  | 0.125  |
| BPY241              | Urine/catheter   | 2            | 0.25                             | 0.125  | 0.125  | 0.125  | 0.125  |
| BPY324              | Sputum           | 4            | 0.25                             | 0.25   | 0.25   | 0.25   | 0.25   |
| BPY373              | BAL fluid        | 2            | 0.25                             | 0.125  | 0.125  | 0.125  | 0.125  |
| BPY376              | Sputum           | 1            | 0.125                            | 0.125  | 0.06   | 1.23   | 0.87   |
| BPY377              | Oral cavity      | 4            | 0.125                            | 0.125  | 0.25   | 0.125  | 0.125  |
| BPY388              | BAL fluid        | 2            | 0.25                             | 0.125  | 0.125  | 0.125  | 0.125  |
| BPY394              | Abscess fluid    | 2            | 0.125                            | 0.25   | 0.06   | 1.05   | 0.79   |
| BPY455              | Urine/catheter   | 4            | 0.5                              | 0.25   | 0.25   | 1.52   | 0.78   |
| BPY458              | Blood            | 8            | 0.5                              | 0.25   | 0.5    | 1.25   | 0.98   |
| BPY480              | Urine/catheter   | 4            | 0.25                             | 0.25   | 0.125  | 1.32   | 0.89   |
| BPY525              | Vaginal          | 2            | 0.125                            | 0.25   | 0.125  | 1.56   | 0.71   |
| BPY531              | Blood            | 2            | 0.125                            | 0.125  | 0.06   | 1.23   | 0.79   |
| BPY544              | Urine/catheter   | 4            | 0.25                             | 0.125  | 0.06   | 0.67   | 0.98   |
| BPY621              | Blood            | 2            | 0.125                            | 0.25   | 0.25   | 0.33   | 0.78   |
| BPY640              | Urine/catheter   | 4            | 0.5                              | 0.125  | 0.25   | 0.54   | 1.12   |

*FLC, fluconazole; ITC, itraconazole; KTC, ketoconazole; VOR, voriconazole. The MICs of these antifungal agents were determined by the broth microdilution method, in accordance with NCCLS document M27-A2 (17). b As specified in footnote c of Table 1. c BAL, bronchoalveolar lavage.

### TABLE 3. Primers and fluorescent probes used in real-time RT-PCR

| Gene (GenBank accession no.) | Primer or probe | Sequencea | Gene location (5’–3’) |
|-----------------------------|----------------|-----------|----------------------|
| CgCDR1 (AF109723)           | CDR1a          | TAGCACATCAACTACACGAACGT | 4500–4522 |
|                             | CDR1b          | AGAGTGAACATTAAGGATGCCCATG | 4647–4670 |
|                             | CDR1pr         | 6FAM-TGCCTGCTTCTTGCCACCTGTT-TAMRA | 4621–4644 |
| CgCDR2 (AF251023)           | CDR2a          | GTGCTTATAGAAGGCTACCAGATT | 164–187 |
|                             | CDR2b          | TCTTAGGGACAGAAGTAACCCTC | 251–274 |
|                             | CDR2pr         | 6FAM-TACCCTTGTGCTGCGGCTGCTCACC-TAMRA | 217–240 |
| CgSNQ2 (AF251022)           | SNQ2a          | ACCATGTGTTCTGAATCATTCAAT | 360–383 |
|                             | SNQ2b          | TCGACATCATTAATGACAGAAA | 462–485 |
|                             | SNQ2pr         | 6FAM-AACACTAATCGCCGAGTTGTGACA-TAMRA | 394–317 |
| ERG11 (L40389)              | ERGa           | ATTTGGGTGTCTTGATGGGTTGTC | 928–949 |
|                             | ERGb           | TCTTCTGGACATCTGGTTCTTCA | 1019–1042 |
|                             | ERGpr          | 6FAM-ACCTTCGGCTGCTACCTCGGCTTGG-TAMRA | 955–978 |
| URA3 (L13661)               | URAa           | GAAAACCAATCTTTTGCTTCTCT | 168–191 |
|                             | URAb           | CATGAGTCTTCAAGCAGAAATGG | 268–291 |
|                             | URApr          | Texas Red-ACGTCACCACCAACAGCAATT-TAMRA | 194–217 |

*a Abbreviations: 6FAM, 6-carboxyfluorescein; TAMRA, 6-carboxy-N,N,N,N-tetramethylrhodamine; Texas Red, product from Molecular Probes; BHQ2, Black Hole Quencher 2.*
conazole, and voriconazole, by the NCCLS method. The resulting susceptibility profiles confirmed those obtained by the screening method. Overall, according to the interpretive criteria for azole susceptibility mentioned above, the 29 _C. glabrata_ isolates were categorized as follows: 20 were resistant (MICs, \( \geq 64 \mu g/ml \)) and 9 were S-DD (MICs, 16 to 32 \( \mu g/ml \)) to fluconazole, 26 were resistant (MICs, \( \geq 1 \mu g/ml \)) and 3 were S-DD (MICs, 0.25 to 0.5 \( \mu g/ml \)) or susceptible (MICs, \( \leq 0.125 \mu g/ml \)) to itraconazole, and 20 were resistant (MICs, \( \geq 1 \mu g/ml \)) and 9 were S-DD (MICs, 0.25 to 0.5 \( \mu g/ml \)) or susceptible (MICs, \( \leq 0.125 \mu g/ml \)) to ketoconazole, and 6 isolates were resistant to one azole (itraconazole).

Genotyping of the 29 isolates showed a very high degree of heterogeneity among them, as 29 unique patterns could be distinguished with probe Cg6 or Cg12, based on differences in the number of bands (from 14 to 4 kb) per isolate, while probe Cg6 hybridized to 9 to 16 different bands (from 14 to 4 kb) per isolate, while probe Cg12 hybridized to 7 to 11 different bands (from 14 to 3.5 kb) per isolate. Thus, all isolates were considered epidemiologically unrelated strains (data not shown).

**Mechanisms of azole resistance in unmatched isolates.** In previous work (12, 22), sets of matched susceptible and resistant isolates, each one derived from a single strain, were used to investigate the molecular mechanisms underlying the development of azole resistance in _Candida_ species. These isolates, however, are rarely available in clinical settings, so it could be of interest to screen for known resistance mechanisms among randomly collected _C. glabrata_ isolates, as shown by a recent investigation with _C. albicans_ (44).

To determine if the present understanding of _C. glabrata_ resistance mechanisms could account for the resistance phenotypes of unmatched clinical isolates, we analyzed the expression of the resistance-associated and non-resistance-associated _C. glabrata_ genes _CgCDR1_, _CgCDR2_, _CgSNQ2_, and _CgERG11_ in our collection of 29 _C. glabrata_ isolates. Expression levels were determined by molecular methods that were also applied to matched sets of isolates. Real-time PCR analysis was performed, and for each target gene, the relative amount of the transcript compared to that of the _URA3_ transcript was determined with total RNA from yeast cells in the logarithmic phase of growth by using a gene-specific fluorescent probe. In addition, a search for point mutations within _CgERG11_ was performed to assess whether this mechanism was responsible for azole resistance in the isolates studied.

(i) _CgCDR1_, _CgCDR2_, and _CgSNQ2_ expression levels. Figure 2 shows the relative quantification of _CgCDR1_, _CgCDR2_, and _CgSNQ2_ (i.e., in reference to that of susceptible control isolate DSY562), expressed as the target gene _R_ normalized to the _URA3_ _R_ for 25 of the 29 isolates included in the study (see Materials and Methods for details). The remaining four isolates were analyzed together with their matched isolates, resulting in pairs of resistant and susceptible isolates (see below for results). In Fig. 2, the results for 25 isolates were separated into two groups on the basis of their levels of susceptibility to fluconazole. The results on the left side of Fig. 2 are for resistant isolates (16 isolates), and those on the right side are for S-DD isolates (9 isolates).

Twelve of the 16 fluconazole-resistant isolates expressed _CgCDR1_ at higher levels than susceptible control isolate DSY562 did, displaying amounts of _CgCDR1_ transcript that increased 11.9- to 263.9-fold compared to those detected in DSY562. Of those isolates that upregulated _CgCDR1_, all were resistant to the other three azoles.

Eight of the 16 fluconazole-resistant isolates expressed _CgCDR2_ at higher levels than the susceptible control isolate DSY562 did, but a lower relative increase (3.8- to 34.7-fold) than that of _CgCDR1_ was observed. Six of the eight isolates that upregulated _CgCDR2_ (isolates BPY46, BPY47, BPY49, BPY50, BPY60, and BPY233) were resistant to the other three azoles, while the remaining two isolates (isolates BPY59 and BPY135) were resistant to two other azoles (ketoconazole and itraconazole).

Eight of the 16 fluconazole-resistant isolates upregulated _CgSNQ2_, but to a lesser extent than they upregulated _CgCDR1_ and _CgCDR2_. The levels of expression in these isolates were 3.7- to 25.3-fold higher than that in susceptible control isolate DSY562. The eight isolates that upregulated _CgSNQ2_ were resistant to the other three azoles (isolates BPY46, BPY48, BPY49, BPY54, BPY55, and BPY60) or two azoles (isolates BPY59 and BPY135).

Overall, nine isolates concomitantly upregulated more efflux pump genes: _CgCDR1_, _CgCDR2_, and _CgSNQ2_ for three isolates (isolates BPY46, BPY49, and BPY60), _CgCDR1_ and _CgCDR2_ for three isolates (isolates BPY47, BPY50, and BPY233), _CgCDR2_ and _CgSNQ2_ for two isolates (isolates BPY59 and BPY135), and _CgCDR1_ and _CgSNQ2_ for one isolate (isolate BPY54).

Interestingly, of the four isolates that did not upregulate _CgCDR1_, two isolates (isolates BPY48 and BPY55) showed a relative increase in the level of expression only of _CgSNQ2_ and were resistant to the four azoles.

The 9 fluconazole S-DD isolates upregulated _CgCDR1_ or _CgCDR2_ or upregulated _CgSNQ2_ (Table 1), but the levels of upregulation were significantly less than those by 16 flucon-
FIG. 2. Expression of CgCDR1, CgCDR2, and CgSNQ2 in the 25 unmatched C. glabrata isolates, as determined by real-time RT-PCR analysis (see the text for details). For each target gene, the relative amount of transcription (i.e., in reference to that by susceptible control isolate DSY562) was compared to that of URA3. The results are reported as the log_{10} fold increases in gene expression levels. Error bars show standard deviations. The x axis indicates the 25 isolates, arranged as resistant (R) and susceptible dose dependent (S-DD) to fluconazole.
azole-resistant isolates (3.7- to 8.0-fold for CgCDR1, 4.7- and 4.8-fold for CgCDR2, and 2.9- to 6.1-fold for CgSNQ2).

(ii) CgERG11 upregulation. In many clinical C. albicans isolates, azole resistance has often been associated with overexpression of the ERG11 gene (12). Likewise, Marichal et al. (14) measured an eightfold increase in ERG11 mRNA levels in an azole-resistant clinical C. glabrata isolate due to amplification of the CgERG11 gene, which in turn resulted from chromosomal duplication. To determine if changes in the levels of expression of CgERG11 could be associated with the resistance phenotype observed in the study isolates, CgERG11 mRNA levels were analyzed (as described above). Beyond expectation, we found that all of the isolates expressed CgERG11 at levels nearly equal to that by susceptible control isolate DSY562.

(iii) CgERG11 sequence analysis. Point mutations in the ERG11 gene with an effect on the affinity of the enzyme for the azoles have been correlated with azole resistance in C. albicans (22, 37, 44). To elucidate if this mechanism could also be implicated in azole resistance in C. glabrata, the CgERG11 genes of all 29 C. glabrata isolates were cloned and sequenced. All the sequences contained at least one silent nucleotide variation compared to the published sequence of CgERG11 (data not shown). Notably, no variation that led to an amino acid substitution was found in any of the isolates.

Development of azole resistance in sequential isolates. As mentioned above, 25 of the 29 C. glabrata isolates in the present study were unmatched isolates recovered from 25 different patients. The medical records of some of the patients, who were mainly hospitalized in critical care units and oncology wards, were retrospectively reviewed and showed that they were persistently infected with C. glabrata. In fact, we noticed the repeated isolation of a unique strain from the same and/or different body sites, as shown by genotyping analyses (data not shown). In contrast, we documented the in vitro development of azole resistance in 4 of the 29 isolates obtained from separate patients with C. glabrata bloodstream infections. For each patient, the first blood culture isolate (fluconazole susceptible) was recovered before the patient was treated with fluconazole. A second isolate (fluconazole resistant) of the same genotype was obtained from the patient after fluconazole therapy was established (Table 1). For the four patients, the times that elapsed between the time of sampling of the azole-susceptible isolate and the time of sampling of the azole-resistant isolate were 15, 35, 40, and 60 days, respectively. At these times, the patients had received cumulative doses of 6, 10, 12, and 12.2 g of fluconazole, respectively. The four pairs of sequential isolates (isolates BPY40 and BPY41, BPY112 and BPY126, BPY241 and BPY285, and BPY449 and BPY479) were studied as described above. As shown in Table 1, isolates BPY41, BPY126, BPY285, and BPY479 all developed marked resistance to the four azoles tested but expressed different resistance profiles. Interestingly, two isolates (isolates BPY285 and BPY479) showed lower rates of resistance to itraconazole (MICs, 2 µg/ml). When the levels of the CgCDR1, CgCDR2, and CgSNQ2 transcripts were measured, all the resistant isolates had a greater abundance of CgCDR1 and CgCDR2 transcripts than the susceptible isolates, while the amount of CgSNQ2 transcript was increased in only two of the four pairs (isolates BPY40 and BPY41 and isolates BPY111 and BPY126) (Fig. 3).

Expression of CgCDR1, CgCDR2, CgSNQ2, and ERG11 in azole-susceptible isolates. We also evaluated the resistance mechanisms cited above for a set of randomly selected clinical isolates of C. glabrata susceptible to azoles and recovered from patients admitted to our hospital during the study period in order to compare their gene expression with that observed for the 29 isolates (fluconazole resistant and S-DD) studied. Table 2 shows the profiles of expression of the CgCDR1, CgCDR2, and CgSNQ2 efflux pump-encoding genes and ERG11, which encodes the azole target, for the 20 susceptible isolates analyzed. As expected, we found that all isolates expressed basal levels of these genes compared to levels of expression by susceptible control isolate DSY562.

Expression of CgCDR1, CgCDR2, CgSNQ2, and ERG11 in isolates grown in the presence of fluconazole. In order to evaluate whether the expression profiles observed for our 20
resistant isolates could change after exposure to fluconazole, we performed experiments in which the expression of the CgCDR1, CgCDR2, CgSNQ2, and ERG11 genes by the isolates grown in the presence of fluconazole at a final concentration of 100 \mu g/ml was analyzed, as described elsewhere (20). The same experiments were also performed with the 24 susceptible isolates, including the 4 isolates of the pairs of sequential isolates, and with the 9 S-DD isolates. The expression levels were compared to those obtained with the same isolates grown in the absence of fluconazole (Tables 4 and 5). Interestingly, the profiles of expression of all target genes, including CgERG11, by the resistant isolates remained unchanged or were only slightly changed, whereas marked increases in the levels of gene expression, particularly of CgCDR1 and CgCDR2, were observed for either the fluconazole-susceptible or S-DD isolates. Similar results were also obtained for the resistant isolates when the isolates were grown in the presence of higher concentrations of fluconazole (200 and 400 \mu g/ml) (data not shown).

**Effects of pump inhibitors on the fluconazole resistance of C. glabrata isolates.** It is known that several compounds are able to reverse the resistance phenotype of yeast strains by inhibiting the drug efflux activity of ABC transporters. Therefore, these compounds are considered potential chemosensitizers for fluconazole-resistant C. glabrata (42). In the present study, we used a functional assay to investigate the effect of the immunosuppressant FK506, the chemosensitizer oligomycin, and the antiarrhythmic drug verapamil on the reversal of fluconazole resistance in our 20 resistant C. glabrata isolates. According to the results of other studies (41, 42), FK506 efficiently reverses the fluconazole resistance of all the pump-overexpressing isolates, as shown by the decreased MICs for the isolates. Instead, oligomycin strongly reduced the fluconazole MICs for susceptible isolates (data not shown). FK506, the chemosensitizer FK506, the chemosensitizer FK506, and the antiarrhythmic drug verapamil on the reversal of fluconazole resistance in our 20 resistant C. glabrata isolates. According to the results of other studies (41, 42), FK506 efficiently reverses the fluconazole resistance of all the pump-overexpressing isolates, as shown by the decreased MICs for the isolates. Instead, oligomycin strongly reduced the fluconazole MICs for susceptible isolates (data not shown).

**DISCUSSION**

Although C. glabrata infection is second or third in frequency, after C. albicans, as a difficult-to-treat infection and is associated with a high mortality rate in at-risk hospitalized patients, very little is known to date about the epidemiology, pathogenesis, treatment, and, above all, antifungal resistance...
of *C. glabrata* isolates (10). A more recent concern has been the numerous reports describing infections due to *C. glabrata* with documented in vitro antifungal resistance in compromised patients taking long-term oral antifungal agents (10, 29, 45, 47). Compared to other *Candida* species, especially *C. albicans*, the MICs of all azoles for *C. glabrata* isolates tend to be higher and *C. glabrata* isolates are intrinsically less susceptible to all antifungal agents, including amphotericin B (10). Although primary in vitro resistance to fluconazole has been reported (31), acquired resistance to azoles is, by far, the most common form of resistance in *C. glabrata* (45–47) and is most often seen for fluconazole. It is conceivable that the selective pressure of fluconazole, due to the increased use of this antifungal in some settings, has contributed to the emergence of *C. glabrata* isolates, especially in isolates that are capable of expressing multiple mechanisms of resistance, as will be discussed below.

In the present study, we attempted to correlate the resistance phenotypes of 29 clinical *C. glabrata* isolates from the survey mentioned above to the molecular basis of azole resistance by evaluating the isolates for the different resistance mechanisms. As already reported (34), enhanced drug efflux is an important mechanism of azole resistance in *C. glabrata* and occurs as a result of the upregulation of multidrug efflux transporter genes, such as *CgCDR1* and *CgCDR2*. Another mechanism of azole resistance in *C. glabrata* involves increased ERG11-dependent ergosterol synthesis due to an eightfold increase in *ERG11* mRNA levels (14). In a recent work, Sanguinetti et al. (35) explored the role of *CgCDR2*, which was found to be identical to the PDH1 gene reported by Miyazaki et al. (15), in the phenomenon of azole resistance. They examined the levels of expression of both *CgCDR1* and *CgCDR2* in an azole-resistant clinical isolate of *C. glabrata* and showed that the upregulation of *CgCDR1* was significant, *CgCDR2* was only moderately expressed in the same strain. On the other hand, when high-frequency azole resistance (HFAR; 2×10^{-4} to 4×10^{-5}) was established from an azole-susceptible strain, both *CgCDR1* and *CgCDR2* were upregulated to high levels. Interestingly, this HFAR was coupled to the loss of mitochon-
As already mentioned, the set of *C. glabrata* isolates used in the present study was analyzed for the well-characterized molecular mechanisms ofazole resistance. First, we examined the expression of *CgCDR1* and *CgCDR2* in our fluconazole-resistant isolates and observed that most isolates upregulated both genes and that the high levels of transcripts correlated with their high-level resistance to fluconazole and other azoles, including voriconazole. When we compared the levels of expression of *CgCDR1* with those of *CgCDR2*, we noticed that *CgCDR1* upregulation was always clearly manifested in isolates that concomitantly expressed both genes, while *CgCDR2* was expressed at moderate levels. These findings add support to the idea that *CgCDR1* is more closely associated withazole resistance than *CgCDR2* (36). The importance of *CgCDR1* and *CgCDR2* in participating in azole resistance in *C. glabrata* was strengthened by the fact that both genes appeared to be upregulated at low levels in isolates that exhibited a fluconazole S-DD phenotype. Nevertheless, no upregulation of *CgCDR1* or *CgCDR2* was observed in two resistant isolates. For these isolates, it was likely that not-yet-characterized multidrug efflux transporters might be involved.

The gene for the CgSNQ2 pump, recently identified by Sanglard et al. (35) to be similar to a portion of an ABC transporter gene, SNQ2, from *Saccharomyces cerevisiae*, has not previously been associated with resistance. Some evidence by Sanglard et al. (35) showed that expression of CgSNQ2 was little affected by azole resistance in clinical and HFA-resistant mutant strains of *C. glabrata*. In our study, a slight upregulation of the CgSNQ2 gene could be detected in part in either fluconazole-resistant or -S-DD isolates. More interestingly, CgSNQ2 accounted for theazole resistance of the four isolates that did not upregulate *CgCDR1*, alone or in combination with *CgCDR2*. In the two isolates mentioned above, in which no appearance of upregulation of *CgCDR1* and *CgCDR2* was found, only CgSNQ2 was upregulated, so relatively large amounts of CgSNQ2 transcripts were detected, and this feature correlated with their phenotype of resistance to multiple azoles. These data support in part the concept that ABC transporter genes with high degrees of similarity, such as *CgCDR1* and *CgCDR2*, can differ in their abilities to confer azole resistance, as already documented for the homologues CDR1 and CDR2 in *C. albicans* (34). On the other hand, genes such as CgSNQ2, for which little involvement in the acquisition of resistance toazole antifungals has been demonstrated, may play a determinant role in this process. This finding needs to be confirmed by further investigation.

Second, in light of the fact that in *C. albicans* clinical isolates decreased susceptibilities to multipleazole derivatives were mainly associated with the upregulation of CDR genes (i.e., CDR1 and CDR2), as well as with the presence of specific point mutations in the ERG11 gene, we examined the role of ERG11 in determining theazole resistance phenotypes of our isolates. Confirming previous results (7), sequencing of CgERG11 and analysis of its expression performed with our isolates showed no alteration or overproduction, favoring the hypothesis that CgERG11 is not involved in theazole resistance of *C. glabrata*. However, one of the two fluconazole-resistant isolates of *C. glabrata* from a patient with oropharyngeal candidiasis studied by Redding et al. (30) showed the upregulation not only of CgCDR1 and CgCDR2 but also of CgERG11. In contrast, the other strain showed no upregulation of the three genes, thus indicating that the development of resistance to fluconazole by *C. glabrata* is a highly varied process involving multiple molecular mechanisms.

To complement the RNA analysis ofazole-resistant clinical isolates, we analyzed the expression of the target genes in 20 randomly selectedazole-susceptible clinical isolates of *C. glabrata*. As expected, the results provided evidence that the levels of expression of *CgCDR1*, *CgCDR2*, CgSNQ2, and CgERG11 in these isolates were always low. On the other hand, we evaluated whether the expression profiling in our 20 resistant isolates was influenced by exposure to fluconazole. Interestingly, we demonstrated that the upregulation of efflux pump-encoding genes was unchanged or was only minimally changed, suggesting that some genetic alteration that perhaps affects a regulatory gene(s) occurred, thereby resulting in unaltered resistance profiles. In support of this, when cultures of 24 susceptible isolates, including 4 pretreatment isolates matched to their corresponding resistant isolates, were treated with fluconazole, we found that all three ABC transporter genes, as well as the ERG11 gene, were markedly upregulated (Table 4). Finally, FK506, which was previously reported to be an inhibitor of ABC pumps (9), reversed the resistance phenotype in our isolates, probably by impairing the drug efflux enzyme activity, although other ABC pump-independent mechanisms, such as an interaction with the calcineurin pathway, could be involved (38).

In summary, the results obtained in the present study emphasize the role of the upregulation of the ABC efflux transporters CgCDR1, CgCDR2, and CgSNQ2 as a major mechanism ofazole resistance in *C. glabrata*. Although this mechanism has not been sufficient to explain the increased level of resistance to fluconazole seen in isolates from 1 of the 20 patients studied by Bennett et al. (5), it proved to be exclusive in our collection of isolates. The multifactorial nature ofazole resistance in *C. glabrata* must not be disregarded, and more comprehensive studies dealing with other possible underlying resistance mechanisms are warranted.

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