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# Relative Contribution of the ABC Transporters Cdr1, Pdh1, and Snq2 to Azole Resistance in *Candida glabrata*

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**ABSTRACT** The utility of the azole antifungals for the treatment of invasive candidiasis is severely hampered by azole resistance in *Candida glabrata*. This resistance is mediated almost exclusively by activating mutations in the zinc cluster transcription factor Pdr1, which controls the genes encoding the multidrug resistance transporters Cdr1, Pdh1, and Snq2. However, the specific relative contributions of these transporters to resistance are not known. To address this question, the *SAT1* flipper method was used to delete *CDR1*, *PDH1*, and *SNQ2* in a strain of *C. glabrata* engineered to carry a clinically relevant activating mutation in *PDR1*. Susceptibility testing was performed according to the CLSI guidelines, with minor modifications, and confirmed with Etest strips. Of the single-transporter-deletion strains, only the *CDR1* deletion resulted in a decreased azole MIC. The deletion of *PDH1* in combination with *CDR1* resulted in a moderate decrease in MIC compared to that observed with the deletion of *CDR1* alone. *SNQ2* deletion only decreased the MIC in the triple-deletion strain in the absence of both *CDR1* and *PDH1*. The deletion of all three transporters in combination decreased the MIC to the level observed in the *PDR1* deletion strains for some, but not all, azoles tested, which indicates that additional Pdr1 targets likely play a minor role in this process. These results indicate that while Cdr1 is the most important Pdr1-mediated multidrug resistance transporter for azole resistance in this clinical isolate, all three of these transporters contribute to its high-level resistance to the azole antifungals.

**KEYWORDS** *Candida glabrata*, antifungal resistance

The ATP-binding cassette (ABC) transporters are a large family of clinically relevant proteins found across prokaryotic and eukaryotic organisms. ABC transporters act as drug efflux pumps in *Candida glabrata*, as well as in the related yeasts *Saccharomyces cerevisiae* and *Candida albicans*. Additional members of the ABC transporter family play a role in lipid homeostasis, which may also influence antifungal treatment, as the drug targets are influenced by lipid content in the cell (1).

In *C. albicans*, the contributions of different mechanisms of resistance to the azole class of antifungals have been well characterized (2). There are two ABC transporters known to contribute to the resistance phenotype in *C. albicans*, Cdr1 and Cdr2. The deletion of each transporter in a clinical isolate with an activating mutation in the gene encoding the transcription factor Tac1, which results in increased expression of *CDR1* and *CDR2*, established Cdr1 as most important in azole resistance (3). In *C. glabrata*, resistance in clinical isolates is due primarily to activating mutations in the transcription factor Pdr1 that lead to increased expression of the genes encoding one of three ABC transporters, Cdr1, Pdh1, or Snq2 (4–9). *CDR1* was originally found to be upregulated in a resistant clinical isolate of *C. glabrata* compared to the susceptible paired isolate. The deletion of *CDR1* in the resistant isolate resulted in increased susceptibility to the level of the susceptible matched isolate. Reintegration of *CDR1* into the deletion mutant

**Received** 23 May 2018 **Returned for modification** 24 June 2018 **Accepted** 13 July 2018

**Accepted manuscript posted online** 23 July 2018

**Citation** Whaley SG, Zhang Q, Caudle KE, Rogers PD. 2018. Relative contribution of the ABC transporters Cdr1, Pdh1, and Snq2 to azole resistance in *Candida glabrata*. Antimicrob Agents Chemother 62:e01070-18. https://doi.org/10.1128/AAC.01070-18.



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**TABLE 1** Azole susceptibilities performed by broth microdilution for selected strains

| Strain | $MIC_{50}$ (μg/ml) by antifungal[a] | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | FLC | | ITC | | KTC | | MIC | | VRC | |
| | 24 h | 48 h | 24 h | 48 h | 24 h | 48 h | 24 h | 48 h | 24 h | 48 h |
| SM1 | 8 | 8 | 0.5 | 0.5 | 0.125 | 0.063 | 0.125 | 0.125 | 0.125 | 0.125 |
| SM3 | 128 | 256 | 32 | 32 | 2 | 4 | 1 | 1 | 4 | 8 |
| SM1RPDR1(SM3) | 128 | 256 | 32 | 32 | 2 | 4 | 1 | 1 | 4 | 8 |
| Δ*cdr1* mutant | 16 | 16 | 1 | 0.5 | 0.25 | 0.25 | 0.125 | 0.25 | 0.125 | 0.25 |
| Δ*pdh1* mutant | 128 | 256 | 32 | 32 | 2 | 4 | 1 | 1 | 4 | 8 |
| Δ*snq2* mutant | 128 | 256 | 32 | 32 | 2 | 4 | 1 | 1 | 4 | 8 |
| Δ*cdr1* Δ*pdh1* mutant | 8 | 8 | 0.125 | 0.125 | ≤0.031 | ≤0.031 | ≤0.016 | 0.063 | 0.031 | 0.063 |
| Δ*cdr1* Δ*snq2* mutant | 16 | 16 | 0.5 | 0.5 | 0.125 | 0.25 | 0.125 | 0.25 | 0.125 | 0.25 |
| Δ*pdh1* Δ*snq2* mutant | 128 | 256 | 32 | 32 | 2 | 4 | 1 | 1 | 4 | 8 |
| Δ*cdr1* Δ*pdh1* Δ*snq2* mutant | 4 | 2 | 0.125 | 0.125 | ≤0.031 | ≤0.031 | ≤0.016 | ≤0.016 | 0.031 | 0.031 |

[a]Values are representative data from experiments completed in duplicate. FLC, fluconazole; ITC, itraconazole; KTC, ketoconazole; MIC, miconazole; VRC, voriconazole.

restored the azole-resistant phenotype (4). *PDH1* was also first implicated when its expression was found to be increased in azole-resistant clinical isolates of *C. glabrata* (6), and further study showed that the deletion of *PDH1* results in increased susceptibility to fluconazole (5). The most recent ABC transporter that has been shown to contribute to azole resistance is Snq2. It was found to have increased expression in two azole-resistant clinical isolates that did not overexpress *CDR1* or *PDH1* (8) and was subsequently shown in one of the same isolates to be required for the azole-resistant phenotype (9).

In order to overcome azole resistance in *C. glabrata*, it is first important to thoroughly understand the mechanisms by which it exhibits resistance to the azoles. While it is known that in *C. glabrata*, the vast majority of clinical resistance has been attributed to upregulation of ABC transporters, the relative importance of each of these remains unclear. Based on previous work in *C. glabrata* and *C. albicans*, we predicted that Cdr1 plays a significant role in azole resistance compared to Pdh1 and Snq2. In this study, we used a strain engineered to carry an activating mutation in *PDR1* from an azole-resistant clinical isolate that results in the upregulation of all three ABC transporters that have been shown to influence azole susceptibility in *C. glabrata*. By generating deletion strains of each transporter alone and in combination, we were able to characterize their individual contributions to the phenotype.

## RESULTS

**Deletion of *CDR1*, but not *PDH1* or *SNQ2*, alone alters fluconazole susceptibility in a resistant *C. glabrata* strain.** The *SAT1* flipper strategy for gene disruption was used to generate deletion strains in *C. glabrata*. Single-gene-deletion mutants were made for each ABC transporter known to contribute to azole resistance (*CDR1*, *SNQ2*, and *PDH1*). The azole-resistant strain SM1RPDR1(SM3) was used as the parent strain for the mutants. This strain was made in our laboratory previously by replacing the *PDR1* allele in fluconazole susceptible-dose-dependent (SDD) clinical isolate SM1 with the *PDR1* allele from azole-resistant isolate SM3, which contains the well-characterized L946S activating mutation (10). Isolate SM1 was recovered from a patient with an abdominal abscess prior to antifungal treatment. SM3 was recovered from an abscess from the same patient 46 days later after a course of fluconazole, followed by caspofungin and then amphotericin B (11).

Susceptibility testing was performed by broth dilution for a panel of azoles, as well as Etest for fluconazole. Both assays confirmed susceptibility data published previously for SM1, SM3, and SM1RPDR1(SM3). The fluconazole MIC for SM1 is 8 μg/ml (Table 1 and Fig. 1A), which would be considered susceptible-dose dependent according to the most recent CLSI breakpoints. The fluconazole MIC for both SM3 and SM1RPDR1(SM3) is 256 μg/ml. The deletion of *CDR1* in strain SM1RPDR1(SM3) resulted in a decreased fluconazole MIC from 256 μg/ml to 16 μg/ml. However, neither *PDH1* nor *SNQ2* single-deletion mutants exhibited a change in fluconazole MIC. Fluconazole Etest

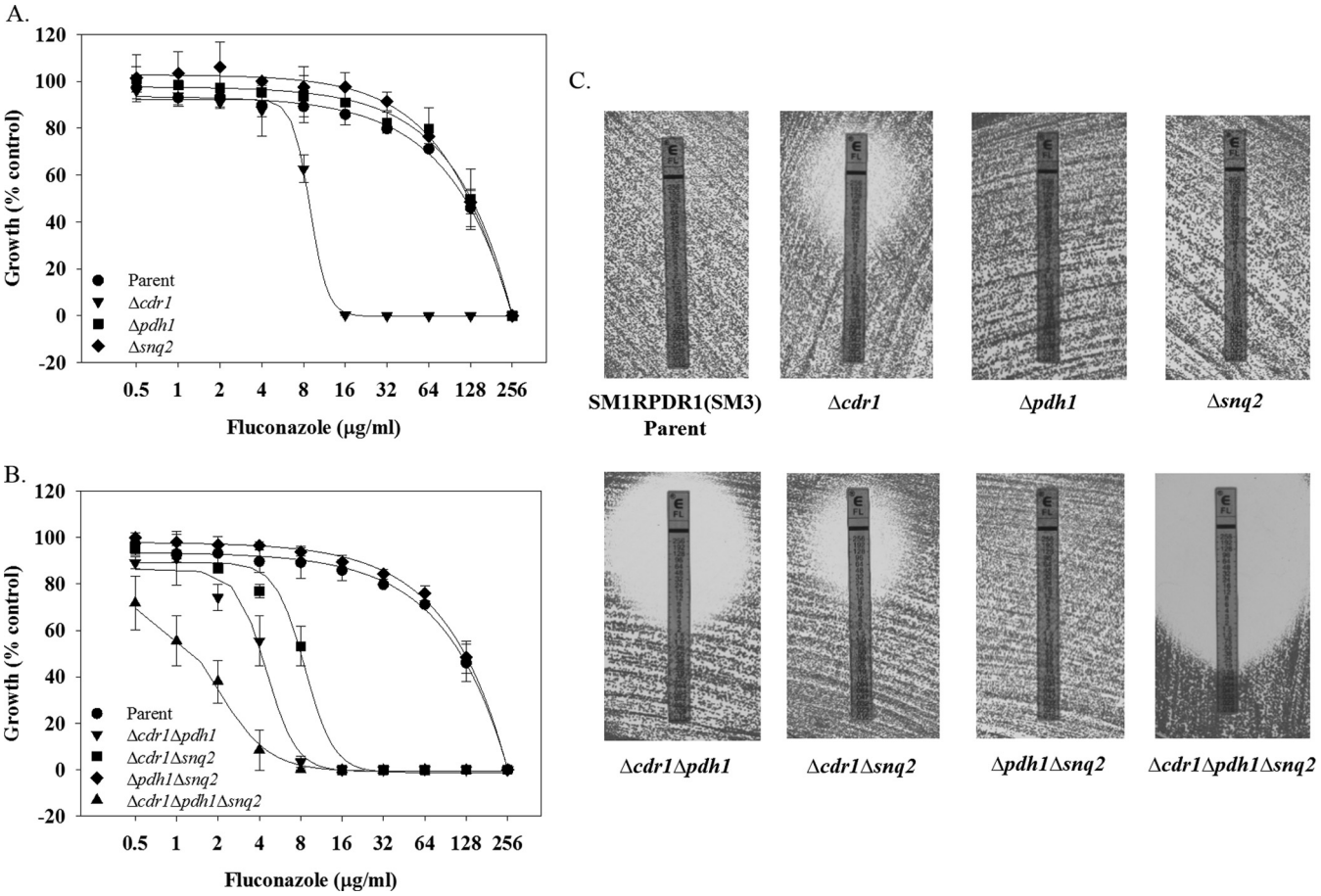


**FIG 1** Altered fluconazole sensitivities in strains lacking the genes encoding the ABC transporters *CDR1*, *PDH1*, and *SNQ2*. (A and B) Fluconazole susceptibilities for the parent and single-deletion strains (A) or the parent, double-, and triple-deletion strains (B) for the ABC transporters *CDR1*, *PDH1*, and *SNQ2*, were measured by broth microdilution as described. Absorbance at 600 nm was measured after incubation at 35°C for 48 h. Background due to medium alone was subtracted, and data were plotted as percentage of the no-drug control. Experiments were completed in triplicate. (C) Susceptibility testing was performed using Etest strips, as described. Images were taken at 48 h. Experiments were performed in duplicate, with representative images shown.

susceptibility testing confirmed the results from the broth microdilution assay (Fig. 1C). The same trend was seen with additional azoles tested (Table 1). Among single-ABC-transporter-deletion mutants, *CDR1* alone was able to alter azole susceptibility.

***PDH1* and *SNQ2* contribute to fluconazole resistance to a lesser extent than *CDR1*.** Previous studies have demonstrated a role for Pdh1 and Snq2 in resistance to azoles. However, we show here that the deletion of *PDH1* or *SNQ2* in an azole-resistant strain overexpressing all three transporters resulted in no change in fluconazole susceptibility (Table 1 and Fig. 1A). In order to determine if *PDH1* and *SNQ2* impact fluconazole resistance in the absence of *CDR1*, double- and triple-deletion strains were made using the *SAT1* flipper method described above.

When *PDH1* and *CDR1* were deleted in combination, the MIC was consistently one doubling dilution lower than the MIC for the strain in which *CDR1* alone is deleted (Table 1 and Fig. 1B). When *SNQ2* and *CDR1* were deleted in combination, the MIC was the same as that observed for the *CDR1* single-deletion strain. To investigate the possibility that the presence of *PDH1* is masking the effect of *SNQ2*, we constructed a strain with all three transporters deleted. The MIC for the triple-deletion strain was consistently one doubling dilution less than that of the *CDR1* and *PDH1* deletion strain. The patterns seen by broth microdilution were confirmed by Etest susceptibility testing (Fig. 1C).

***CDR1*, *PDH1*, and *SNQ2* mutants exhibit similar susceptibility patterns to other azole antifungals.** Of interest is the potential for these transporters to affect members

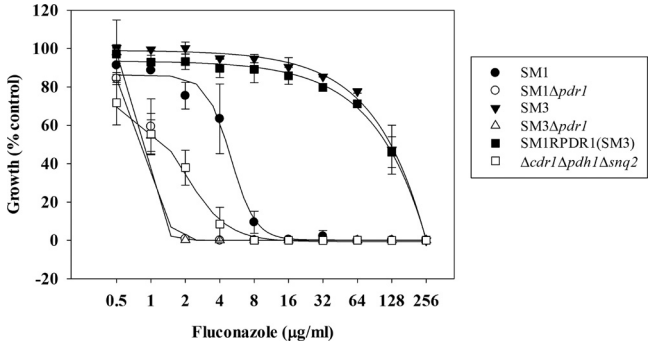


**FIG 2** Altered fluconazole sensitivities to strains lacking the genes encoding all three relevant ABC transporters compared to strains lacking the gene encoding the transcription factor Pdr1. Fluconazole susceptibility was measured by broth microdilution, as described. Absorbance at 600 nm was measured after incubation at 35°C for 48 h. Background due to medium alone was subtracted, and data were plotted as percentage of the no-drug control. Experiments were performed in triplicate.

of the azole class of antifungals differently. Newer triazoles in the class have improved spectra of activity against additional fungal pathogens. The differences in chemical structure could influence the interaction with efflux pumps, resulting in different ABC transporters preferentially effluxing different azoles. However, overall, the patterns for all azoles tested are similar, with few differences (Table 1). The magnitude of changes in MIC are different among the azoles for the different ABC transporters. In general, however, the differences from the parent strain and the triple-ABC-transporter-deletion strain were 6 to 8 drug dilutions for all azoles tested. Among the single-deletion mutants, Cdr1 was the only transporter able to influence the MIC for all azoles tested. Interestingly, the deletion of *SNQ2* had no impact on itraconazole or ketoconazole MIC. No notable differences were observed in the susceptibilities to echinocandins and amphotericin B in the mutant strains (data not shown).

***CDR1*, *PDH1*, and *SNQ2* explain most, but not all, of Pdr1's contribution to azole resistance.** All three ABC transporters in this study are known targets of Pdr1. All three transporters are upregulated in strains that possess an activating mutation in *PDR1* (9, 10, 12). Pdr1 has also been shown to bind to the promoter regions of each transporter by chromatin immunoprecipitation sequencing (ChIP-seq) (13). In order to determine if these three transporters account for the entirety of Pdr1's importance to azole resistance, we compared *PDR1* deletion strains to the triple-ABC-transporter-deletion strain. Both the susceptible-dose-dependent and resistant clinical isolates lacking *PDR1* exhibit increased sensitivity to fluconazole with a treatment of 2 $\mu$g/ml, resulting in $>$95% growth reduction (Fig. 2). The triple-deletion mutant, however, required treatment with 8 $\mu$g/ml fluconazole to exhibit a similar reduction in growth. This indicates that there may be additional genes of the Pdr1 regulon that contribute to azole resistance.

## DISCUSSION

Activating mutations in the zinc cluster transcription factor Pdr1 that result in upregulation of ABC transporters are found in the vast majority of azole-resistant clinical isolates of *C. glabrata*. Similarly to the transcription factors Pdr1 and Pdr3 in the closely related yeast *S. cerevisiae* (14), Pdr1 in *C. glabrata* exerts its regulatory effects by binding a pleiotropic drug response element (PDRE) (13, 15). Pdr1 is also autoregulated due to a PDRE in its promoter region (15). Developing a better understanding of this mechanism of azole resistance is important for discovering new treatment strategies. For example, a small molecule that could inhibit efflux by the ABC transporters would allow clinicians to once again successfully treat *C. glabrata* with azoles.

Many different activating mutations have been found throughout the coding sequence for *PDR1* that result in similar decreases in azole susceptibility (10, 12, 15–18). However, there are differences in the gene expression patterns of the Pdr1 regulon for the various mutations. Of particular interest are the patterns of expression of the three

ABC transporters known to influence azole susceptibility, which do not appear to correlate with the location of the mutation (12).

ABC transporter expression profiles in clinical isolates reveal a wide distribution of expression patterns. *CDR1*, *PDH1*, or both *CDR1* and *PDH1* were found to be highly expressed in 18 of 20 resistant clinical isolates from one study. The 2 remaining resistant isolates overexpressed *SNQ2* alone. The susceptible-dose-dependent isolates overexpressed the ABC transporters to a lesser extent than the resistant isolates (8). Another group showed that 10 of 12 resistant clinical isolates and 6 of 7 laboratory-generated resistant mutants exhibited increased expression of *CDR1* or both *CDR1* and *PDH1* (19). A third study of resistant clinical isolates found *CDR1* or *PDH1* overexpression in the resistant isolate of 12 of 14 matched clinical isolates (20). A study in which the expression of *CDR1*, *PDH1*, and *PDR1* was measured in a panel of clinical isolates, with the purpose of developing a quantitative PCR (qPCR)-based assay for the determination of resistance in *C. glabrata*, found that *CDR1* expression alone could be used to predict resistance with 100% sensitivity and 95% specificity. The same was not true for *PDH1* expression or *PDR1* expression (21).

One might predict the other ABC transporters to overcome the absence of one or two transporters and maintain a high level of resistance. *C. albicans*, which has only two ABC transporters that have been shown to contribute to the azole-resistant phenotype, does not exhibit compensatory upregulation of the other ABC transporter when one is absent (3). In *S. cerevisiae*, the deletion of *SNQ2* and *YOR1* leads to enhanced activity of fluconazole and ketoconazole; however, the *SNQ2-YOR1-PDR5* triple-deletion strain demonstrates enhanced sensitivity to the azoles tested. Protein and mRNA levels of expression indicate that there is compensatory upregulation with different combinations of these ABC transporter deletion mutants (22).

The phenomena seen in this study with the deletion of *PDH1* and *SNQ2* not having a phenotypic effect in the presence of *CDR1* or *CDR1* and *PDH1*, respectively, suggests a possible interaction between these ABC transporters. Work in *S. cerevisiae* would indicate that this is likely. Membrane yeast two-hybrid (MYTH) technology demonstrated a physical interaction between Pdr5 (homolog for *C. glabrata* Cdr1) and Snq2; however, no changes in protein level, transcript level, or localization of Snq2 in the *PDR5* deletion strain were detected (23). Snq2 and Pdr5 in *S. cerevisiae* have also demonstrated a synergistic effect on estradiol transport. The deletion of *SNQ2* alone has no effect on estradiol accumulation, but when deleted in conjunction with *PDR5*, there is a dramatic increase in intracellular estradiol (24). Pdr5 and Pdr15 (homolog for *C. glabrata* Pdh1) showed similar additive sensitivity to 2,4-dichlorophenol and various membrane-altering nonionic detergents, such that the double-deletion strains demonstrated marked increased sensitivity compared to the single-deletion strains (25).

All three ABC transporters are found in the plasma membrane. The alteration of their expression alone would affect the composition of the plasma membrane and potentially alter azole uptake in this way. The deletion of *PDR5* and *PDR15* in *S. cerevisiae* results in an increase in the amount of phosphatidylethanolamine exposed in the plasma membrane (25). The mechanism by which azoles are taken up into the cell is not well understood at this time, but alteration of the membrane could influence this as-yet-unknown process.

Our experiments show that the three ABC transporters previously implicated in Pdr1-mediated azole resistance in *C. glabrata* are not able to fully explain the decrease in susceptibility in the absence of Pdr1. In addition to controlling the expression of ABC transporters, Pdr1 also regulates the expression of many other genes both directly and indirectly. We predict that there likely are genes among this group that make small contributions to azole resistance that in combination may explain the phenotypic difference between the *PDR1* deletion strain and the triple-ABC-transporter-deletion strain.

Among the list of genes whose promoters were found to be directly bound by Pdr1 are a major facilitator superfamily (MFS) transporter, *QDR2*, and two additional ABC transporters, *YBT1* and *YOR1* (13). The deletion of *QDR2* in *C. glabrata* resulted in

**TABLE 2** Strains used in this study

| Strain | Parent | Genotype or description | Reference |
|---|---|---|---|
| SM1 | | Azole-SDD clinical isolate | 11 |
| SM3 | | Azole-resistant clinical isolate | 11 |
| SM1RPDR1(SM3) | SM1 Δ*pdr1* | *pdr1*Δ::*FRT-PDR1*$^{SM3}$ | 10 |
| S1RPS3CDR1M2A and S1RPS3CDR1M2B | SM1RPDR1(SM3) | *pdr1*Δ::*FRT-PDR1*$^{SM3}$/*cdr1*Δ::*FRT* | This study |
| S1RPS3PDH1M2A and S1RPS3PDH1M2B | SM1RPDR1(SM3) | *pdr1*Δ::*FRT-PDR1*$^{SM3}$/*pdh1*Δ::*FRT* | This study |
| S1RPS3SNQ2M2A and S1RPS3SNQ2M2B | SM1RPDR1(SM3) | *pdr1*Δ::*FRT-PDR1*$^{SM3}$/*snq2*Δ::*FRT* | This study |
| S1RPS3CAPDH1M2A | S1RPS3CDR1M2A | *pdr1*Δ::*FRT-PDR1*$^{SM3}$/*cdr1*Δ::*FRT*/*pdh1*Δ::*FRT* | This study |
| S1RPS3CBPDH1M2A | S1RPS3CDR1M2B | *pdr1*Δ::*FRT-PDR1*$^{SM3}$/*cdr1*Δ::*FRT*/*pdh1*Δ::*FRT* | This study |
| S1RPS3CASNQ2M2A | S1RPS3CDR1M2A | *pdr1*Δ::*FRT-PDR1*$^{SM3}$/*cdr1*Δ::*FRT*/*snq2*Δ::*FRT* | This study |
| S1RPS3CBSNQ2M2A | S1RPS3CDR1M2B | *pdr1*Δ::*FRT-PDR1*$^{SM3}$/*cdr1*Δ::*FRT*/*snq2*Δ::*FRT* | This study |
| S1RPS3SAPDH1M2A | S1RPS3SNQ2M2A | *pdr1*Δ::*FRT-PDR1*$^{SM3}$/*pdh1*Δ::*FRT*/*snq2*Δ::*FRT* | This study |
| S1RPS3SBPDH1M2A | S1RPS3SNQ2M2B | *pdr1*Δ::*FRT-PDR1*$^{SM3}$/*pdh1*Δ::*FRT*/*snq2*Δ::*FRT* | This study |
| S1RPS3CAPASNQ2M2A | S1RPS3CAPDH1M2A | *pdr1*Δ::*FRT-PDR1*$^{SM3}$/*cdr1*Δ::*FRT*/*pdh1*Δ::*FRT*/*snq2*Δ::*FRT* | This study |
| S1RPS3CBPASNQ2M2A | S1RPS3CBPDH1M2A | *pdr1*Δ::*FRT-PDR1*$^{SM3}$/*cdr1*Δ::*FRT*/*pdh1*Δ::*FRT*/*snq2*Δ::*FRT* | This study |

increased susceptibility to the imidazoles, clotrimazole, ketoconazole, and miconazole but had no effect on fluconazole or itraconazole susceptibility (26). *YOR1* and *YBT1* are frequently found among genes with altered expression in relation to azole susceptibility, but a direct relationship has not been described. The effects of *QDR2*, *YOR1*, and *YBT1* on fluconazole susceptibility could potentially be masked by the presence of the more dominant transporters, *CDR1*, *PDH1*, and *SNQ2*, and therefore cannot be ruled out as contributing to azole resistance, albeit likely a minor contribution.

Other genes of interest shown previously to be upregulated in clinical isolates (10, 17) and laboratory-derived strains (27) of *C. glabrata* with activating mutations in *PDR1* are *RSB1* and *RTA1*. Rsb1 is a putative sphingolipid flippase. The homolog for Rta1 in *S. cerevisiae* is a member of the fungal lipid-translocating exporter family of proteins. The promoter regions of both *RSB1* and *RTA1* possess a PDRE that is bound directly by Pdr1 (15). Recent work in *C. albicans* has implicated the putative lipid translocase encoded by *RTA3* and the sphingolipid flippase encoded by *RTA2* in azole resistance (28, 29). Rsb1 and Rta1 may make similar contributions to azole resistance in *C. glabrata*.

## MATERIALS AND METHODS

**Strains and growth media.** All strains used in this study are listed in Table 2. The clinical isolates and the *PDR1* replacement strain have been described previously (10, 11). All strains were stored as frozen stocks at −80°C with 40% glycerol. Strains were routinely grown in YPD broth (1% yeast extract, 2% peptone, and 2% dextrose) at 30°C in a shaking incubator, except as indicated for specific experimental conditions.

*Escherichia coli* PX5-α chemically competent cells (Protein Express, Cincinnati, OH) were used as the host for plasmid construction and propagation. These strains were grown at 37°C in Luria-Bertani (LB) broth or on LB plates supplemented with 100 μg/ml ampicillin (Sigma, St. Louis, MO) or 50 μg/ml kanamycin (Fisher BioReagents, Fair Lawn, NJ).

**Plasmid construction.** For the deletion of *CDR1*, *PDH1*, and *SNQ2*, we modified plasmid pSFS2 (30). Upstream homology regions approximately 800 to 1,000 bp long were amplified using primer pairs CgCDR1A/CgCDR1B, CgPDH1/CgPDH1B, or CgSNQ2A/CgSNQ2B and digested with ApaI/XhoI or KpnI/ApaI as indicated by underlined bases for insertion into their respective plasmids. Downstream homology regions approximately 800 to 1,000 bp long were amplified using primer pairs CgCDR1C/CgCDR1D, CgPDH1C/CgPDH1D, or CgSNQ2C/CgSNQ2D and digested with SacII/SacI or NotI/SacII as indicated by underlined bases for insertion into their respective plasmids. The disruption cassettes consisting of the *SAT1* flipper cassette and upstream and downstream flanking sequences of either *CDR1*, *PDH1*, or *SNQ2* were excised from the final plasmid pCgCDR1, pCgPDH1, or pCgSNQ2 and gel purified. The primers used to construct the cassettes are listed in Table 3.

**Strain construction.** *C. glabrata* cells were transformed by the lithium acetate method using approximately 1 μg of DNA. The ApaI/SacI fragments from pCgCDR1 and pCgSNQ2 and the KpnI/SacII fragment from pCgPDH1 were excised and gel purified prior to transformation. The transformed cells were allowed to recover for 6 h in YPD at 30°C before being plated on YPD agar plates containing 200 μg/ml nourseothricin (Jena Biochemical, Germany) and incubated at 30°C. Positive transformants were selected within 24 h, and successful insertion of the disruption cassette at the target gene locus was confirmed by Southern hybridization using gene-specific probes. Subsequently, induction of the flipper recombinase gene in the disruption cassette was performed by overnight growth of the positive transformant clones in YPD at 30°C with shaking (under no selective pressure). Selection for excision of the *SAT1* flipper cassette was then performed by plating on YPD agar plates and incubating for up to 24 h at 30°C. Clones were selected and confirmed by Southern hybridization using gene-specific probes.

**TABLE 3** Primers used in this study

| Primer name | Primer sequence (5′–3′)[a] |
|---|---|
| CgCDR1A | CATAGATCAGGGCCCATTACATTAGCACAG |
| CgCDR1B | CTCAGTGTTGCTCGAGATAGGGTTGATAC |
| CgCDR1C | GTTCTGTTAGTTCCGCGGACTCTCGTAGAT |
| CgCDR1D | GTGAATACAAACAAGAGCTCCACAATAATA |
| CgPDH1A | AAACAGTCTATGGTACCACAAGTTTGCACA |
| CgPDH1B | CCGTATACGTTTCGGGCCCTTGTCATCAA |
| CgPDH1C | ACAGAAGATGCGGCCGCTATGGTATATTTATT |
| CgPDH1D | ATTCCTTAATAACCGCGGAAGTTGACTTTA |
| CgSNQ2A | TTGAGTATCTTAGGGCCCTTGTTTTCAGTT |
| CgSNQ2B | GATAGAATACTCGAGTTGTCGCTGTGCGC |
| CgSNQ2C | GCTATTTATTACCGCGGCCATGTCAGAG |
| CgSNQ2D | AGACAGATATTGAGCTCCACTACTGCTGAG |

[a]Underlined bases indicate the introduction of restriction enzyme cloning sites to allow directional cloning into the *SAT1*-flipper cassette.

**Isolation of genomic DNA and Southern hybridization.** Genomic DNA from *C. glabrata* was isolated as described previously (31). For confirmation by Southern hybridization, approximately 10 $\mu$g of genomic DNA was digested with the appropriate restriction enzymes, separated on a 1% agarose gel containing ethidium bromide, transferred by vacuum blotting onto a nylon membrane, and fixed by UV-cross-linking. Hybridization was performed with the Amersham AlkPhos direct labeling and detection system (GE Healthcare, Pittsburgh, PA), as per the manufacturer's instructions.

**Susceptibility testing.** Susceptibility testing was performed by broth microdilution assay and by Epsilometer test strips (Etest). Broth microdilution was performed according to the CLSI guidelines outlined in document M27-A3, with a few modifications (32). A fluconazole (MP Biomedicals, Solon, OH) stock solution was prepared by reconstitution in water to 5 mg/ml. Itraconazole (0.5 mg/ml), ketoconazole (10 mg/ml), miconazole (10 mg/ml), and voriconazole (10 mg/ml) (Sigma, St. Louis, MO) stock solutions were prepared by reconstitution in dimethyl sulfoxide (DMSO; Sigma) at the indicated concentrations. Colonies grown overnight on Sabouraud dextrose plates were diluted to $2.5 \times 10^3$ cells/ml in RPMI 1640 (Sigma) with 2% glucose and morpholinepropanesulfonic acid (MOPS) (pH 7.0). Plates were incubated at 35°C for 48 h. Absorbance at 600 nm was read with a BioTek Synergy 2 microplate reader (BioTek, Winooski, VT); background due to medium was subtracted from all readings. The MIC was defined as the lowest concentration inhibiting growth by at least 50% relative to the drug-free control after incubation with drug for 24 or 48 h. Fluconazole broth microdilution susceptibility testing was performed in triplicate, and additional azole broth microdilution susceptibility testing was performed in duplicate. Fluconazole Etest (bioMérieux) susceptibility assay was performed as per the manufacturer's instructions, with some modifications, in triplicate. Colonies were selected from cultures grown overnight on Sabouraud dextrose plates and diluted in water to a 0.5 McFarland standard. Sterile cotton swabs were used to streak plates prior to Etest strip placement. Plates were incubated at 35°C and read at 24 and 48 h.

## ACKNOWLEDGMENTS

This work was supported by NIH NIAID grants R01AI058145 (to P.D.R.) and R01AI131620 (to P.D.R.).

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