

UPC2A Is Required for High-Level Azole Antifungal Resistance in *Candida glabrata*

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Candida glabrata, the second most common cause of *Candida* infections, is associated with high rates of mortality and often exhibits resistance to the azole class of antifungal agents. Upc2 and Ecm22 in *Saccharomyces cerevisiae* and Upc2 in *Candida albicans* are the transcriptional regulators of *ERG11*, the gene encoding the target of azoles in the ergosterol biosynthesis pathway. Recently two homologs for these transcription factors, *UPC2A* and *UPC2B*, were identified in *C. glabrata*. One of these, *UPC2A*, was shown to influence azole susceptibility. We hypothesized that due to the global role for Upc2 in sterol biosynthesis in *S. cerevisiae* and *C. albicans*, disruption of *UPC2A* would enhance the activity of fluconazole in both azole-susceptible dose-dependent (SDD) and -resistant *C. glabrata* clinical isolates. To test this hypothesis, we constructed mutants with disruptions in *UPC2A* and *UPC2B* alone and in combination in a matched pair of clinical azole-SDD and -resistant isolates. Disruption of *UPC2A* in both the SDD and resistant isolates resulted in increased susceptibility to sterol biosynthesis inhibitors, including a reduction in fluconazole MIC and minimum fungicidal concentration, enhanced azole activity by time-kill analysis, a decrease in ergosterol content, and downregulation of baseline and inducible expression of several sterol biosynthesis genes. Our results indicate that Upc2A is a key regulator of ergosterol biosynthesis and is essential for resistance to sterol biosynthesis inhibitors in *C. glabrata*. Therefore, the *UPC2A* pathway may represent a potential cotherapeutic target for enhancing azole activity against this organism.

Fungal infections caused by opportunistic organisms have continued to increase in recent decades and have become an important medical concern (1–3). *Candida glabrata* in particular has emerged over the past 2 decades as the predominant cause of yeast infections in diabetics (54%) and the elderly (51%) and is second to *Candida albicans* in most other patient populations (4–7). Candidemia mortality rates continue to rise, with rates for *C. glabrata* reported as high as 50% (8–10). The emergence of *C. glabrata* as a common pathogen is complicated by, and likely related to, its intrinsically low susceptibility to azole antifungals and its ability to rapidly develop high-level azole resistance during treatment (11–14).

Ergosterol is an essential component of the fungal cell membrane and is an important signaling molecule in the cell. It helps maintain membrane integrity and fluidity, which facilitates several membrane-bound enzymatic reactions. Ergosterol is not synthesized by the host, so the ergosterol biosynthesis pathway has long been a target for antifungal agents (Fig. 1). Compounds that inhibit this pathway are broadly categorized as sterol biosynthesis inhibitors (SBIs). The azole class of antifungals specifically targets lanosterol 14- α -demethylase (Erg11), which catalyzes the C-14 demethylation of lanosterol (15–17). Statins, such as lovastatin, inhibit the gene products of *HMG1* and *HMG2*, which convert 3-hydroxy-3-methyl-glutaryl coenzyme A (HMG-CoA) to mevalonate, resulting in a reduction in the synthesis of cholesterol in mammals and the synthesis of ergosterol in yeasts (Fig. 1).

As has been observed in *Saccharomyces cerevisiae* and *C. albicans*, exposure to azoles also results in upregulation of genes from the ergosterol biosynthesis pathway (including *ERG11*) in *C. glabrata* (14–17). The predominant mechanism of azole resistance in *C. glabrata* is the constitutive overexpression of ATP-binding

cassette (ABC) transporter genes, *CDR1*, *PDH1*, and *SNQ2*, which are under the transcriptional regulation of a hyperactive form of the transcription factor Pdr1. *ERG* gene expression and regulation could also be important in acquired azole resistance, as is the case in *C. albicans*, or may potentially contribute to the intrinsic reduced azole susceptibility in *C. glabrata* (14, 18–25).

Resistant isolates of *S. cerevisiae* often have increased expression of *ERG11*, as well as other ergosterol biosynthesis genes, which are under the control of the transcriptional regulators Upc2 and Ecm22 (26, 27). In *C. albicans*, a single homolog, Upc2, has been identified and found to be involved in *ERG* gene regulation (17, 25, 28–30). These transcriptional regulators are members of the well-characterized fungus-specific Zn₂-Cys₆ family of transcriptional activators (18). *ERG11* is not an essential gene in *C. glabrata*. Disruption of this gene or pharmacologic inhibition of its product prevents ergosterol production, which causes accumulation of alternate sterols, including lanosterol, obtusifoliol, and 4,14 α -dimethyl zymosterol, leading to decreased susceptibility to

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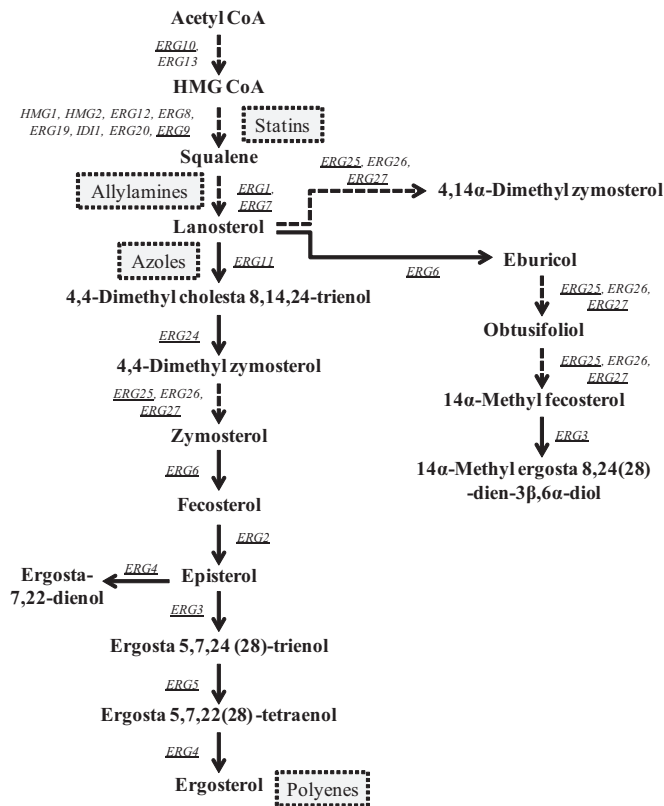


FIG 1 Representation of the ergosterol biosynthesis pathway in *Candida glabrata*. Genes (in italics) whose expression was monitored in this study are underlined. Sites of action for specific drug classes are indicated by dashed box. CoA, coenzyme A.

fluconazole, itraconazole, and amphotericin B (31, 32). Biosynthesis of these alternate sterols does not require *ERG11* but does require other genes in the ergosterol biosynthesis pathway.

Nagi et al. recently described two *Upc2* homologs in *C. glabrata*, *Upc2A* and *Upc2B*. *Upc2A* was shown to play a major role in the regulation of *ERG2* and *ERG3* in the ergosterol biosynthesis pathway (24). A strain with a disruption of *UPC2A* also exhibited 4-fold and 16-fold increases in fluconazole and lovastatin (a member of the statin class of cholesterol-lowering agents) susceptibilities, respectively (24). Given the global role of *Upc2* in regulation of sterol biosynthesis, we reasoned that disruption of

UPC2A might enhance the activity of fluconazole in azole-resistant as well as azole-susceptible dose-dependent (SDD) clinical isolates.

MATERIALS AND METHODS

Strains and growth media. The matched fluconazole-SDD and -resistant clinical isolate set used in this study has been described previously (12, 19). All strains (Table 1) were maintained in YPD (1% yeast extract, 2% peptone, and 2% dextrose) broth at 30°C and stored as 40% glycerol stocks at -80°C. One Shot *Escherichia coli* TOP10 chemically competent cells (Invitrogen, Carlsbad, CA) 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 of ampicillin (Sigma, St. Louis, MO) or 50 µg/ml of kanamycin (Fisher BioReagents, Fair Lawn, NJ).

Gene disruption. Mutant strains (Table 1) were generated by the *SAT1*-flipper gene disruption method (33). Briefly, approximately 900 bp of sequence immediately upstream of the target gene plus the first 50 bp of the open reading frame (ORF), referred to as the 5' flanking region, was cloned upstream of the *SAT1*-flipper cassette in pSFS2A, and the final 50 bp of the target gene ORF plus approximately 900 bp of sequence immediately downstream of the target gene, referred to as the 3' flanking region, was cloned downstream of the *SAT1*-flipper cassette. The disruption cassettes consisting of the *SAT1*-flipper and 5' and 3' flanking sequences of either the *UPC2A* or *UPC2B* genes were excised from the final plasmid construct and gel purified. Primers used to construct the cassettes are listed in Table 2. *C. glabrata* cells were transformed by the lithium acetate method using approximately 1 µg of DNA. 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 of 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. 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 of cassette excision was then performed by plating a series of dilutions of this culture on YPD agar plates containing 25 µg/ml of nourseothricin and incubating them for up to 24 h at 30°C. Clones were selected due to size differences (slower-growing colonies usually indicated successful cassette excision) and confirmed by Southern hybridization (see Fig. S1 in the supplemental material).

Likewise, reintroduction of the *UPC2A* gene into the *UPC2A* locus was accomplished as described above with a *SAT1*-flipper cassette in which the 5' flanking sequence cloned upstream of the flipper cassette was replaced with a DNA fragment consisting of the 5' flanking sequence and full-length ORF of *UPC2A*. Isolate-specific constructs were prepared to reintroduce the native *UPC2A* alleles back into their original locus. This cassette was excised, gel purified, and used for cell transformation as

TABLE 1 Strains used in this study

Strain	Parent	Genotype or description	Reference
SM1		Azole-SDD clinical isolate	12
SM1Δ <i>upc2A</i>	SM1	<i>upc2A</i> Δ::FRT	This study
SM1Δ <i>upc2B</i>	SM1	<i>upc2B</i> Δ::FRT	This study
SM1Δ <i>upc2A</i> Δ <i>upc2B</i>	SM1	<i>upc2A</i> Δ::FRT/ <i>upc2B</i> Δ::FRT	This study
SM1Δ <i>upc2A</i> / <i>UPC2A</i>	SM1	<i>upc2A</i> Δ::FRT- <i>UPC2A</i>	This study
SM1Δ <i>upc2A</i> Δ <i>upc2B</i> / <i>UPC2A</i>	SM1	<i>upc2A</i> Δ::FRT- <i>UPC2A</i> / <i>upc2B</i> Δ::FRT	This study
SM3		Azole-resistant clinical isolate	12
SM3Δ <i>upc2A</i>	SM3	<i>upc2A</i> Δ::FRT	This study
SM3Δ <i>upc2B</i>	SM3	<i>upc2B</i> Δ::FRT	This study
SM3Δ <i>upc2A</i> Δ <i>upc2B</i>	SM3	<i>upc2A</i> Δ::FRT/ <i>upc2B</i> Δ::FRT	This study
SM3Δ <i>upc2A</i> / <i>UPC2A</i>	SM3	<i>upc2A</i> Δ::FRT- <i>UPC2A</i>	This study
SM3Δ <i>upc2A</i> Δ <i>upc2B</i> / <i>UPC2A</i>	SM3	<i>upc2A</i> Δ::FRT- <i>UPC2A</i> / <i>upc2B</i> Δ::FRT	This study

TABLE 2 Primers used in this study (grouped by application)

Primer function and name	Primer sequence ^a
Cassettes for constructing mutants	
CgUPC2A-A	5'-GTTAAAACGGGCCCATGATCGTCGTATCC-3'
CgUPC2A-B	5'-AATAGCTTTGGCTCGGTATCCTCTATGT-3'
CgUPC2A-C	5'-CTAGAGATGCGGCCGCTTATAGTGGTGGT-3'
CgUPC2A-D	5'-TATCGATTTAAACCGCGGTGATATTCTTCCA-3'
CgUPC2A-E	5'-GTACCTAGTGGGCCACCTTGATCTGT-3'
CgUPC2A-F	5'-AATAAAGGAAGTAAATTCGATATTTTCAGAG-3'
CgUPC2B-A	5'-TGAGTTCAGTTTTATGGGCCCTTAGAAAG-3'
CgUPC2B-B	5'-AGTTGACTAACCTTCTTACCTCGAGTATCTA-3'
CgUPC2B-C	5'-TAAGATATTGCCGCGGTTACTAACGATGT-3'
CgUPC2B-D	5'-CAAACTATAGGAGCTCGAAGGAATATGTG-3'
qRT-PCR	
ACT1F	5'-CGCTTTGGACTTCGAACAAGAA-3'
ACT1R	5'-GTTACCGATGGTGATGACTTGAC-3'
ERG1F	5'-GGTAAAGAGGTGCTGATTG-3'
ERG1R	5'-GATGTTGTTGATGGACTGG-3'
ERG2F	5'-AATCTGCTATTGCTGGTTGCC-3'
ERG2R	5'-CGCTGCCGTTGTGTTGG-3'
ERG3F	5'-AAGCGTGTGAACAAGGAC-3'
ERG3R	5'-TACAATAGCAGACCGAAGAC-3'
ERG4F	5'-GCTGTACGCTAACGCTTGTG-3'
ERG4R	5'-CAGTGGCAGTATGTGTATGGAAC-3'
ERG5F	5'-CCCAGCCCTACACGACCCAGAAG-3'
ERG5R	5'-GGACCACGCCGAAGACCAACC-3'
ERG6F	5'-TCCTCTTTCCACTTCTCCCGTTTC-3'
ERG6R	5'-GCCACCGACACCGCAACC-3'
ERG7F	5'-ATGCCTGATGGTGGTTGG-3'
ERG7R	5'-TCTTCATTGGACTTAGCC-3'
ERG9F	5'-CAGCACCAATGACACCAG-3'
ERG9R	5'-CCTCAGAATCAGATAGAAGACC-3'
ERG10F	5'-GTTATCGCTGGTGGTTGTG-3'
ERG10R	5'-CATTGGTGGTGGTGCCTAAG-3'
ERG11F	5'-TACCAAGCCATACGAGTTC-3'
ERG11R	5'-GGTCAAGTGGGAGTAAGC-3'
ERG24F	5'-ACTCGCTGGACTACTCTTC-3'
ERG24R	5'-CAACTTAGTGGCTCTCTTAG-3'
ERG25F	5'-ATGTGTATGGATTACCTTGAGAC-3'
ERG25R	5'-GTAGTACCAGTGAAGTAGTGG-3'
ERG27F	5'-CCAGGAAAGTAGCCGTTATCAC-3'
ERG27R	5'-TCAACTCAACAACCTCTTACAC-3'
18SF	5'-TCGGCACCTTACGAGAAATCA-3'
18SR	5'-CGACCATACTCCCCCAGA-3'
PDR1F	5'-TTTGACTCTGTTATGAGCGATTACG-3'
PDR1R	5'-TTCGGATTTTCTGTGACAATGG-3'
CDR1F	5'-CATACAAGAAACACAAAGTCGGT-3'
CDR1R	5'-GAGACACGCTTACGTTACCAC-3'
SNQ2F	5'-CGTCTATGTCTTCTTACACCATT-3'
SNQ2R	5'-TTTGAACCGCTTTGTCTCTGA-3'
PDH1F	5'-ACGAGGAGGAAGACGACTACGA-3'
PDH1R	5'-CTTACTGGAACTCATCGTGGT-3'

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

described above. Screening for positive clones was accomplished as described above (see Fig. S1).

Isolation of genomic DNA and Southern hybridization. Genomic DNA from *C. glabrata* was isolated as described previously (34). For confirmation by Southern hybridization, approximately 10 µg 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 ECL direct nucleic acid labeling and detection system (GE Healthcare, Pittsburgh, PA) as per the manufacturer's instructions.

Susceptibility testing. Susceptibility testing was performed by broth microdilution assay according to the CLSI guidelines outlined in document M27-A3, with a few modifications (35). Antifungal agents were obtained from the following sources: fluconazole was from LKT Laboratories (St. Paul, MN), miconazole was from MP Biomedicals, Inc. (Solon, OH), anidulafungin and voriconazole were from Pfizer (New York, NY), and ketoconazole, amphotericin B, lovastatin, and terbinafine were from Sigma (St. Louis, MO). All drugs were dissolved in dimethyl sulfoxide

(DMSO) and diluted to yield a final DMSO concentration of ≤0.5% in all experiments. Growth of *C. glabrata* was not affected at this concentration of DMSO (data not shown). Cultures were diluted to 2.5×10^3 cells/ml in RPMI medium (Sigma) with 2% glucose and morpholinepropanesulfonic acid (MOPS), pH 7.0. Plates were incubated at 35°C for 24, 48, and 72 h. Absorbance at 620 nm was read with a microplate reader (Beckman Coulter, Inc., Fullerton, CA); background due to medium was subtracted from all readings. The MIC was defined as the lowest concentration inhibiting growth by at least 80% (or no visible growth for amphotericin B) relative to that of the drug-free control after incubation with drug for 24 h. While CLSI guidelines indicate an endpoint of 50% inhibition of growth, we chose to use a more stringent endpoint of 80% inhibition of growth in order to detect a more pronounced effect. Absorbance measured at 72 h was used to assess regrowth of the cultures (36). The minimum fungicidal concentration (MFC) was determined by spotting 5 µl from each well of the 48-h MIC plate onto YPD solid media and incubating the media at 30°C for an additional 24 h. The MFC was defined as the lowest concentration that exhibited complete inhibition of growth (37, 38).

Spot assays. Colonies from cultures grown on Sabouraud dextrose (BD Biosciences, San Jose, CA) solid media for 24 h were diluted in sterile water to an optical density at 600 nm of 0.1. Cultures were serially diluted one to four in sterile water. Two microliters of each dilution was spotted on each of three solid media: YPD, YPD with 5 µg/ml of fluconazole, and YPD with 10 µg/ml of fluconazole. The serial dilutions were incubated at 30°C for 24 and 72 h. Plates were imaged at each time point, and degrees of growth inhibition were compared (20, 36, 39).

Time-kill analysis. Time-kill experiments were adapted from the methods described by Klepser et al. (40). Briefly, isolates started from freezer stocks were subcultured twice on potato dextrose agar (BD Biosciences, San Jose, CA). Colonies were picked from the plates into sterile water and the densities adjusted to that of a 0.5 McFarland standard. The suspensions were then diluted 1:10 in RPMI medium to a final volume of 3 ml with or without 10 µg/ml of fluconazole. Cultures were incubated with agitation at 35°C. One-hundred-microliter samples were removed from each culture at 0, 6, 12, and 24 h and serially diluted in sterile water, and 50-µl aliquots were spread on potato dextrose agar. Plates were incubated at 35°C and colonies counted at 24 and 48 h. Each experiment was performed in triplicate.

Ergosterol quantification analysis. Ergosterol was extracted and quantified as described by Arthington-Skaggs et al. (41). Briefly, a single colony of *C. glabrata* from a fresh YPD plate was used to inoculate 50 ml of RPMI medium (Sigma, St. Louis, MO) and incubated for 16 h with shaking at 35°C. Stationary-phase cells were collected by centrifugation for 5 min at 2,700 rpm and washed twice with sterile distilled water. The net weight of the pellet was determined. To each pellet, 3 ml of 25% alcoholic potassium hydroxide solution (25 g of KOH and 35 ml of sterile distilled water brought up to 100 ml with 100% ethanol) was added, followed by vortexing for 1 min. Cell suspensions were transferred to a sterile borosilicate glass screw-cap tube and incubated at 85°C for 1 h. Sterols were then extracted from cool tubes by the addition of a mixture of 3 ml of *n*-heptane and 1 ml of sterile distilled water, followed by vortexing for 3 min. The heptane layer was transferred to a clean sterile borosilicate glass tube and stored at -20°C for up to 24 h. One hundred microliters of sterol-heptane mixture was scanned spectrophotometrically between 240 nm and 300 nm with a DU530 Life Sciences UV spectrophotometer (Beckman Coulter, Brea, CA). The presence of ergosterol in the extracted sample resulted in a characteristic four-peak curve with peaks located at approximately 262, 270, 281, and 290 nm. The absence of detectable ergosterol was indicated by a flat line. *n*-Heptane was used to blank the spectrophotometer. A decrease in the height of the absorbance peaks correlated to a decrease in ergosterol content. Statistical significance was determined by using Student's *t* test ($P < 0.05$).

RNA isolation. For RNA analysis, log-phase cultures grown in YPD medium at 30°C were adjusted to an optical density of 0.2 measured at 600 nm. Various concentrations of drug or the DMSO control were added,

TABLE 3 Broth microdilution susceptibility testing of the susceptible dose-dependent clinical parent isolate SM1, mutant strains with disruptions of *UPC2*, and a revertant

Strain	MIC ($\mu\text{g/ml}$) ^a								
	FLC	ITC	VRC	KTC	MC	AMB	LOVA	TRB	ANID
SM1	8	2	2	2	0.5	2	200	512	0.016
SM1 Δ <i>upc2A</i>	0.5	0.0078	0.125	0.0625	0.01563	2	25	32	0.016
SM1 Δ <i>upc2B</i>	8	1	1	0.5	0.25	2	256	512	0.016
SM1 Δ <i>upc2A</i> Δ <i>upc2B</i>	0.5	0.0078	0.125	0.01563	0.01563	2	16	8	0.016
SM1 Δ <i>upc2A</i> / <i>UPC2A</i>	8	2	2	2	0.5	2	200	512	0.016

^a FLC, fluconazole; ITC, itraconazole; VRC, voriconazole; KTC, ketoconazole; MC, miconazole; AMB, amphotericin B; LOVA, lovastatin; TRB, terbinafine; ANID, anidulafungin.

and the cultures were incubated for an additional 3 h to mid-log phase. RNA was extracted by the hot-phenol method (42), as previously described (43). RNA was treated with RQ1 DNase (Promega, Madison, WI). Quantity and purity were determined with a spectrophotometer (NanoDrop Technologies, Inc., Wilmington, DE).

Quantitative RT-PCR analysis. Quantitative real-time PCR (RT-PCR) was conducted as described previously (43). Single-strand cDNA was synthesized from 2 μg of total RNA using the SuperScript First Strand Synthesis System for RT-PCR (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions. Relative quantitative real-time PCRs were performed in triplicate using the ABI Prism 7000 sequence detection system (Applied Biosystems, Foster City, CA). Independent PCRs were performed using the primers listed in Table 2 for both the genes of interest and either 18S rRNA or *ACT1* using SYBR green PCR master mix (Applied Biosystems). Relative gene expression was calculated by the comparative threshold cycle ($\Delta\Delta C_T$) method. For expression of *PDR1*, *CDR1*, *SNQ2*, and *PDH1*, samples were normalized first to 18S rRNA expression and then to the untreated SM1 sample. For expression of genes in the ergosterol biosynthesis pathway, samples were normalized first to *ACT1* and then to the untreated parent sample, SM1 or SM3.

RESULTS

Disruption of *CgUPC2A* results in increased susceptibility to azole antifungals and other sterol biosynthesis inhibitors in *C. glabrata*.

Using the *C. glabrata* genome database maintained by Génolevures (www.genolevures.org/cagl.html), BLASTP analysis was performed to search for homologs of either ScUpc2 or ScEcm22. Two potential homologs were identified, CAGL0C01199g and CAGL0F07865g, which were recently reported by Nagi et al. as *UPC2A* and *UPC2B*, respectively (24). *UPC2A* (CAGL0C01199g) alone, *UPC2B* (CAGL0F07865g) alone, and both genes together were disrupted in fluconazole-SDD clinical isolate, SM1, and tested against fluconazole (Table 3). As observed by Nagi et al., the disruption of *UPC2A*, alone or in combination with disruption of *UPC2B*, showed a marked increase in susceptibility to fluconazole, whereas the disruption of *UPC2B* alone did not. Complementation of *UPC2A* back into its native locus restored wild-type susceptibility. In addition to fluconazole, strains were tested against a panel of azoles, nonazole SBIs (terbinafine and lovastatin), and antifungal agents not involved in sterol biosynthesis (amphotericin B and anidulafungin) (Table 3). The additional azoles tested exhibited susceptibility patterns similar to that observed with fluconazole. Terbinafine inhibits squalene epoxidase and lovastatin inhibits HMG-CoA reductase, both early enzymes in the ergosterol biosynthesis pathway (Fig. 1). As was observed with the azole antifungals, strains with disruptions of *UPC2A* exhibited increased susceptibility to both of these agents (Table 3). As reported previously for *C. albicans* (29), disruption of either *UPC2A* or *UPC2B* had no effect on susceptibility to amphotericin B, which binds

ergosterol in the fungal cell membrane. Likewise, disruption of *UPC2* had no effect on susceptibility to the echinocandin anidulafungin, which affects cell wall stability by targeting β -1,3-glucan synthase (Table 3). Finally, reintroduction of *UPC2A* was able to restore wild-type susceptibilities. These results demonstrate that *UPC2A* influences the susceptibility of *C. glabrata* to a range of clinically used azoles as well as other SBIs.

Disruption of *UPC2A* results in enhanced activity of fluconazole against *C. glabrata*. Fluconazole remains the most widely used antifungal agent prescribed for the treatment of *Candida* infections (44). We therefore wished to further investigate the extent to which *UPC2A* influences fluconazole susceptibility in *C. glabrata*. In isolate SM1, disruption of *UPC2A* alone or in combination with disruption of *UPC2B* resulted in a reduction not only in the fluconazole MIC (Table 3) but also in the fluconazole MFC, whereas disruption of *UPC2B* had no effect on either of these parameters (Fig. 2A). This phenotype reverted to that of the parent, SM1, when *UPC2A* was reintroduced. We used a 72-h endpoint for a broth microdilution assay in RPMI medium as a way to assess the ability of the organism to resume growth in the presence of fluconazole. The *UPC2A* mutant was unable to resume growth at this extended time point, whereas isolate SM1 was able to resume growth in concentrations up to 8 $\mu\text{g/ml}$ (Fig. 2B). Serial dilutions of the strains with disruptions of *UPC2A* spotted on YPD solid medium containing 5 $\mu\text{g/ml}$ or 10 $\mu\text{g/ml}$ of fluconazole exhibited reduced growth, while SM1 Δ *upc2B* and the *UPC2A* revertants were no different than the parent, SM1. Moreover, the cultures were unable to recover after incubation for 72 h (Fig. 2C). Likewise, by time-kill analysis, disruption of *UPC2A* in isolate SM1 resulted in greater inhibition by 10 $\mu\text{g/ml}$ of fluconazole than for the wild-type isolate (Fig. 2D). These results demonstrate that disruption of *UPC2A* greatly influences the ability of *C. glabrata* to grow in the presence of fluconazole.

***UPC2A* is essential for resistance to sterol biosynthesis inhibitors in an azole-resistant clinical isolate of *C. glabrata*.** In order to determine if *UPC2A* or *UPC2B* influences resistance to SBIs, we disrupted these genes in a clinical isolate that exhibits high-level azole resistance due to overexpression of the *CDR1*, *PDH1*, and *SNQ2* transporter genes as a result of an activating mutation in the transcription factor gene *PDR1*. The resulting isolate, SM3, was collected from the same patient as isolate SM1 after azole treatment failure and is described in detail elsewhere (12, 19). SM3 exhibited elevated MICs compared to those of SM1 for fluconazole, itraconazole, voriconazole, ketoconazole, and miconazole, as well as lovastatin and terbinafine. As was observed with isolate SM1, disruption of *UPC2A* alone or in combination with *UPC2B* resulted in increased suscepti-

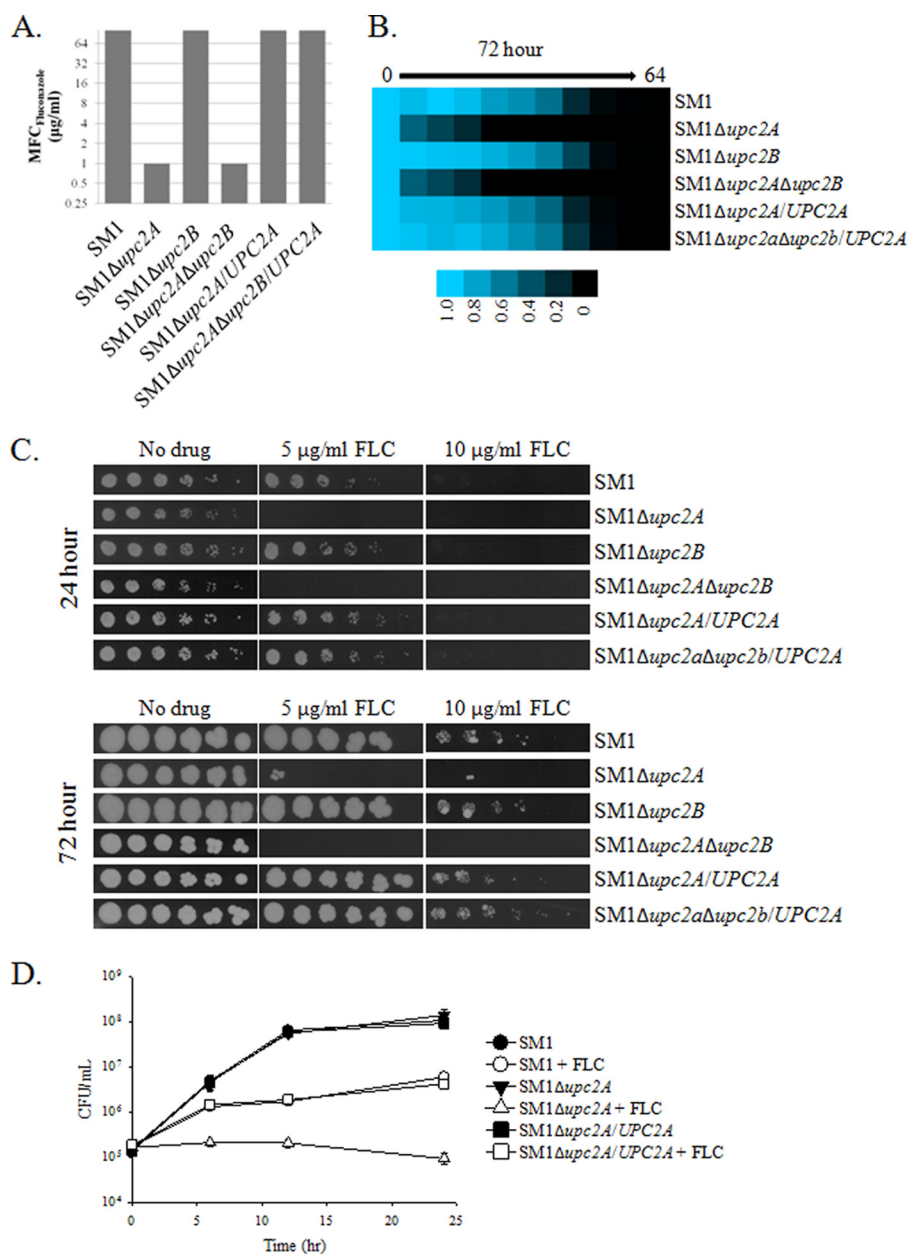


FIG 2 Enhanced activity of fluconazole in an SDD clinical isolate, SM1, with a disruption of *UPC2A*. (A) Minimum fungicidal concentrations were determined by spotting cultures on solid media after 48 h of growth in liquid RPMI medium. The MFC is the lowest concentration of fluconazole that inhibited growth completely when the strain was transferred to solid medium. (B) Broth microdilution assays were conducted according to CLSI guidelines. As a measure of the ability of the strains to regrow, the plates were allowed to incubate for 72 h prior to measurement of the optical density at 600 nm. (C) Serial dilutions of the indicated strains were incubated for 24 or 72 h on YPD solid media containing 0, 5, or 10 µg/ml of fluconazole. (D) Time-kill curves for *UPC2A* disruptants and revertants of a susceptible clinical isolate, SM1, treated with 10 µg/ml of fluconazole (open symbols) or a DMSO control (closed symbols). Cultures were grown in RPMI medium, with aliquots removed at the indicated time points and plated on potato dextrose agar for colony counts.

bility to all azoles and sterol biosynthesis inhibitors tested (Table 4). Indeed, disruption of *UPC2A* reduced the susceptibility of fluconazole to that observed in the wild-type isolate SM1, 8 µg/ml. Fluconazole MFCs of the strains lacking *UPC2A* decreased to 16 µg/ml, compared to >256 µg/ml for isolate SM3 and the revertants (Fig. 3A). After 72 h in RPMI medium, growth of this strain was unable to resume at a fluconazole concentration of ≥ 16 µg/ml (Fig. 3B). When serial dilutions of *UPC2A* disruptant strains were spotted on solid YPD medium

containing 10 µg/ml of fluconazole, growth was inhibited (Fig. 3C). The growth inhibition was maintained after incubation for 72 h. By time-kill analysis, fluconazole concentrations of 10 µg/ml had no effect on isolate SM3 after 24 h, whereas disruption of *UPC2A* resulted in approximately a log fold reduction in growth after 24 h of fluconazole treatment (Fig. 3D). This was roughly equivalent to the effect of fluconazole on isolate SM1. These results demonstrate that *UPC2A* is essential for high-level fluconazole resistance in *C. glabrata*.

TABLE 4 Broth microdilution susceptibility testing of the resistant clinical isolate SM3, mutant strains with disruptions of *UPC2*, and a revertant

Strain	MIC ($\mu\text{g/ml}$) ^a								
	FLC	ITC	VRC	KTC	MC	AMB	LOVA	TRB	ANID
SM3	256	8	8	8	2	2	200	512	0.016
SM3 Δ <i>upc2A</i>	8	0.125	0.25	0.25	0.062	2	25	32	0.016
SM3 Δ <i>upc2B</i>	256	16	4	8	2	2	256	512	0.016
SM3 Δ <i>upc2A</i> Δ <i>upc2B</i>	8	0.25	0.25	0.25	0.062	2	64	16	0.016
SM3 Δ <i>upc2A/UPC2A</i>	256	8	8	8	2	2	200	512	0.016

^a FLC, fluconazole; ITC, itraconazole; VRC, voriconazole; KTC, ketoconazole; MC, miconazole; AMB, amphotericin B; LOVA, lovastatin; TRB, terbinafine; ANID, anidulafungin.

Disruption of *UPC2A* does not affect expression of *PDR1*, *CDR1*, *SNQ2*, or *PDH1* in an azole-resistant clinical isolate of *C. glabrata*. We observed that disruption of *UPC2A* increased susceptibility to fluconazole not only in clinical isolate SM1 but also in matched azole-resistant isolate SM3, which carries an activating mutation in *PDR1* and overexpresses *CDR1* and *PDH1*, as well as *PDR1* itself. Recently, CaUpc2 was shown to bind *in vivo* to the promoter of *CDR1*, suggesting that *C. glabrata* Cdr1 may also be a direct target of CgUpc2A (45, 46). Those authors found that CaUpc2 is involved in maintaining baseline expression of *CDR1* in *C. albicans* and observed a slight decrease in *CDR1* expression in the absence of *UPC2*.

To determine what role, if any, the deletion of *UPC2A* in *C. glabrata* has on the expression of the efflux pump genes *CDR1*, *PDH1*, *SNQ2*, and *PDR1*, we examined the expression of these genes by qRT-PCR in strains SM1 and SM3, as well as their respective mutants with disruptions of *UPC2A* and the revertants grown for 3 h with or without 32 $\mu\text{g/ml}$ of fluconazole. Disruption of *UPC2A* had no effect on the expression of these genes in the absence of fluconazole (Fig. 4). However, in fluconazole-treated cells, we did observe a reduction in the upregulation of all of the genes tested in the SM1 Δ *upc2A* mutant strain compared to the wild type (Fig. 4). Moreover, disruption of *UPC2A* in isolate SM3 had no effect on the constitutive overexpression of *CDR1*, *PDH1*, or *SNQ2*, suggesting that the reduced azole resistance observed in SM3 upon disruption of *UPC2A* is independent of these transporters.

Disruption of *UPC2A* results in a reduction in ergosterol levels in azole-SDD and -resistant *C. glabrata* isolates. Upc2 has previously been shown to be involved in the regulation of sterol biosynthesis and uptake in both *S. cerevisiae* and *C. albicans* (17, 27). To determine whether the observed decrease in susceptibility to SBIs corresponds to a decrease in ergosterol biosynthesis, we determined the effect of disruption of *UPC2A* and *UPC2B* on total ergosterol content in clinical isolates SM1 and SM3. Sterols were extracted from the mutant and parent strains and measured at an absorption spectrum between 240 and 300 nm as described previously by Arthington-Skaggs et al., who demonstrated a direct correlation between ergosterol content and azole susceptibility in *S. cerevisiae* (41). As shown in Fig. 5, disruption of *UPC2A* alone or in combination with *UPC2B* resulted in a reduction in ergosterol levels in both azole-SDD isolate SM1 and azole-resistant isolate SM3. These results suggest that ergosterol biosynthesis is impaired in both SDD and resistant isolates of *C. glabrata* in the absence of *UPC2A*.

***UPC2* is essential for both baseline and sterol biosynthesis inhibitor-induced expression of ergosterol biosynthesis genes.** Disruption of *UPC2A* resulted in increased susceptibility to SBIs and a significant decrease in total ergosterol content. In order to

determine if disruption of *UPC2A* influences baseline expression of genes involved in sterol biosynthesis, 13 *ERG* genes were examined by qRT-PCR. Disruption of *UPC2A* in both SM1 and SM3 resulted in ≥ 2 -fold downregulation of all *ERG* genes examined. Reintroduction of *UPC2A* into its native locus partially or completely restored expression of most *ERG* genes (Fig. 6). There was no significant difference between SM1 and SM3 in expression of any of the *ERG* genes tested.

Multiple fungal pathogens, including *C. glabrata*, upregulate ergosterol biosynthesis genes in response to treatment with SBIs, which likely contributes to the decreased susceptibility observed with these agents (24, 47). We used qRT-PCR to determine the effects of SBI treatment on strains with disruptions of *UPC2A*. Expression of *ERG* genes was measured for SM1 and SM3, as well as their respective *UPC2A* mutants and revertants, grown for 2 h in the presence or absence of 50 $\mu\text{g/ml}$ of lovastatin or 128 $\mu\text{g/ml}$ of fluconazole.

Similar to the case with *S. cerevisiae* (48), treatment of isolates SM1 and SM3 with lovastatin resulted in ≥ 2 -fold upregulation of 12 of the 13 *ERG* genes examined in SM1 and all of the *ERG* genes examined in SM3. Treatment with fluconazole resulted in upregulation of all of these genes in SM1 and 10 of the 13 *ERG* genes examined in SM3. In the absence of *UPC2A*, lovastatin and fluconazole were unable to induce the expression of any of these *ERG* genes. Moreover, the majority of the 13 *ERG* genes were downregulated ≥ 2 -fold even in the presence of drug treatment. In most cases, reintroduction of *UPC2A* into its native locus in both SM1 and SM3 restored the ability of SBI treatment to induce *ERG* gene expression (Fig. 6). These data demonstrate that *UPC2A* is required for both baseline and SBI induction of *ERG* gene expression in *C. glabrata*.

DISCUSSION

Despite the more recent development of the lipid-based formulations of amphotericin B and the echinocandins, fluconazole remains the most prescribed antifungal agent for the treatment of *Candida* infections. It is inexpensive, available as both intravenous and oral formulations, and associated with minimal adverse effects. Although its use is complicated by many drug-drug interactions, these are well understood and somewhat predictable. Indeed, fluconazole maintains excellent activity against many common and clinically important *Candida* species, such as *C. albicans*, *C. parapsilosis*, and *C. tropicalis* (49). Of particular concern, however, is *C. glabrata*, which has emerged as the second leading cause of candidiasis in the United States and which exhibits intrinsically low susceptibility to the azole antifungals (3, 50–55).

Furthermore, the development of high-level resistance to the azoles is common in *C. glabrata* and has been well documented for

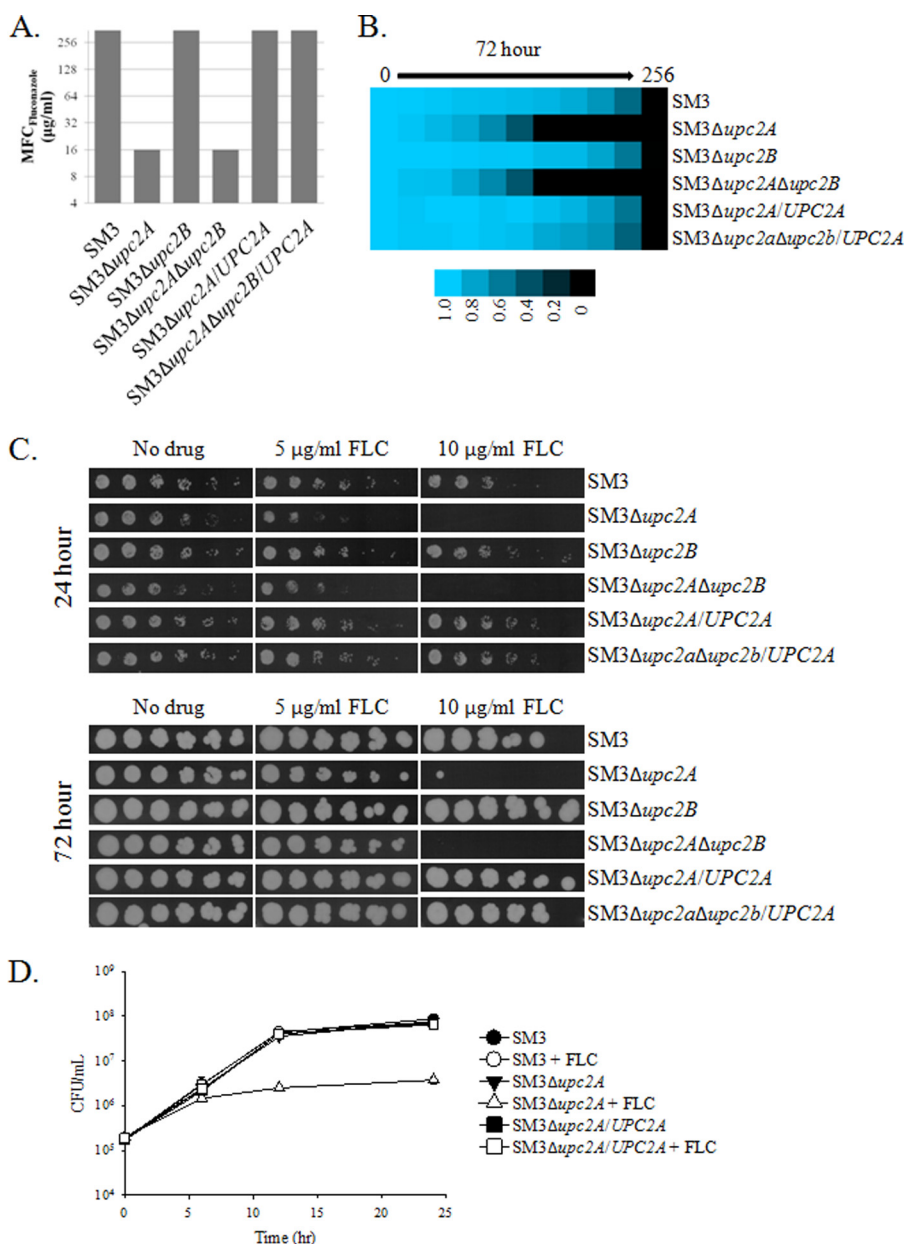


FIG 3 Enhanced activity of fluconazole in a resistant clinical isolate, SM3, with a disruption of *UPC2A*. (A) Minimum fungicidal concentrations were determined by spotting cultures on solid media after 48 h of growth in liquid RPMI medium. The MFC is the lowest concentration of fluconazole that inhibited growth completely when the strain was transferred to solid medium. (B) Broth microdilution assays conducted according to CLSI guidelines. As a measure of the ability of the strains to regrow, the plates were allowed to incubate for 72 h prior to measurement of the optical density at 600 nm. (C) Serial dilutions of the indicated strains were incubated for 24 or 72 h on YPD solid media containing 0, 5, or 10 $\mu\text{g/ml}$ of fluconazole. (D) Time-kill curves for *UPC2A* disruptants and revertants of a resistant clinical isolate, SM3, treated with 10 $\mu\text{g/ml}$ of fluconazole (open symbols) or a DMSO control (closed symbols). Cultures were grown in RPMI media, with aliquots removed at the indicated time points and plated on potato dextrose agar for colony counts.

isolates from head and neck radiation patients, stem cell transplant patients, and human immunodeficiency virus (HIV) patients and for vulvovaginal candidiasis (11, 13, 39, 56, 57). The development of azole resistance has also been implicated in the fluconazole treatment failure and death of a patient suffering from *C. glabrata* candidemia (12). Such resistance appears to be almost exclusively due to activating mutations in the gene encoding the transcription factor Pdr1, leading to overexpression of multidrug resistance transporters Cdr1, Pdh1, and Snq2 (58–60). Strategies

to preserve this antifungal class for use against *C. glabrata* by both enhancing activity and overcoming resistance are needed.

The azoles exert their antifungal action by binding to and inhibiting the activity of the cytochrome P450 enzyme, lanosterol demethylase, which is a key enzyme in the ergosterol biosynthesis pathway that is encoded by *ERG11*. In both *S. cerevisiae* and *C. albicans*, the zinc cluster transcription factor Upc2 has been shown to be a central regulator of not only *ERG11* but also additional genes in the ergosterol biosynthesis pathway. When lanosterol de-

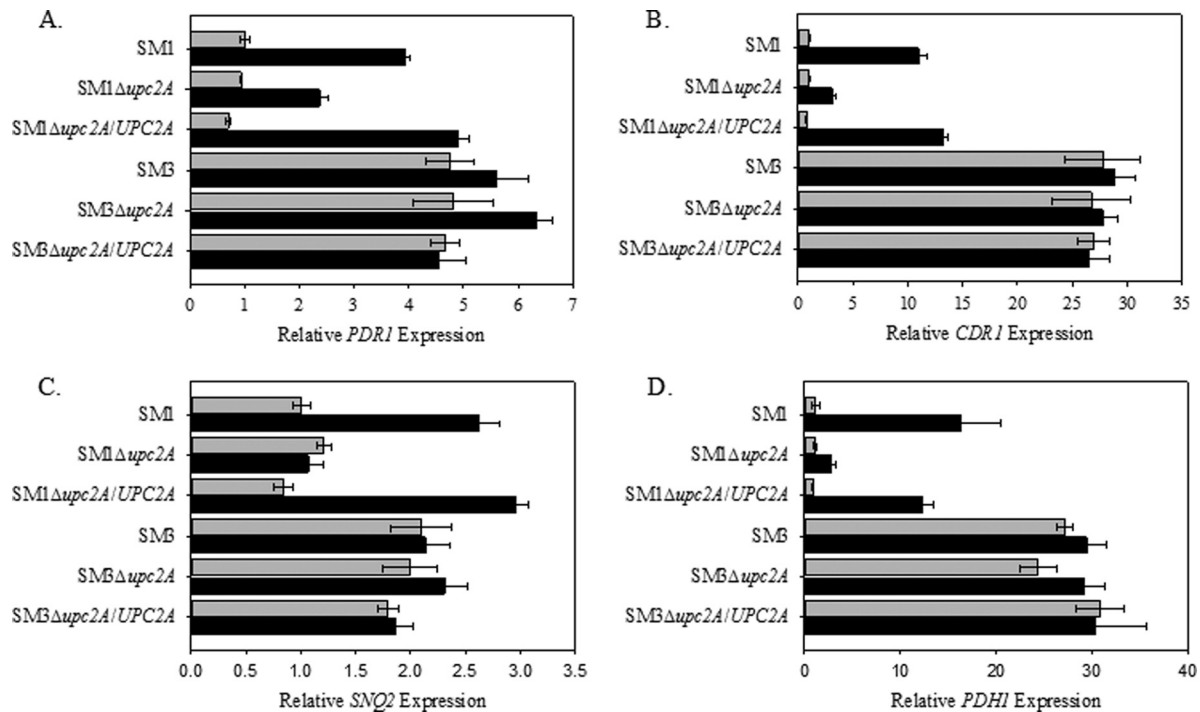


FIG 4 Effect of *UPC2A* disruption on constitutive and fluconazole-induced gene expression. RNA was isolated from mid-log-phase cultures of parent clinical isolates, $\Delta upc2A$ mutants, and revertants, and qRT-PCR analysis of relative gene expression was performed. Light bars represent untreated controls and dark bars represent treatment with 32 $\mu\text{g/ml}$ of fluconazole for 3 h. (A) *PDR1*; (B) *CDR1*; (C) *SNQ2*; (D) *PDH1*. Data are shown as means \pm SEs ($n = 3$).

methylase is inhibited genetically or pharmacologically in *C. glabrata*, alternate sterols and toxic sterol precursors such as lanosterol, 4,14 α -dimethyl zymosterol, and 14 α -methyl ergosta 8,24(28)-dien-3 β ,6 α -diol are produced, leading to reduced growth (31, 32) (Fig. 1). In *S. cerevisiae*, *C. albicans*, and *C. glabrata*, *Upc2* has been shown to be a central regulator of baseline and inducible expression of the genes encoding enzymes of the ergosterol biosynthesis pathway (15, 24, 27, 29, 61). Moreover, disruption of *UPC2* has been shown to increase the susceptibility of these organisms to the azole antifungals (24, 61, 62).

Given its global influence on sterol biosynthesis, we hypothesized that disruption of *Upc2* might enhance the activity of the azole antifungals against azole-resistant as well as azole-SDD clinical isolates of *C. glabrata*. We have recently characterized a matched pair of azole-SDD and -resistant *C. glabrata* clinical isolates in which high-level azole resistance developed during fluconazole treatment (19). The resistant isolate in the pair carries an activating mutation in *PDR1* leading to the overexpression of *CDR1*, *PDH1*, and *SNQ2* and an increase in fluconazole MIC from 8 $\mu\text{g/ml}$ to 256 $\mu\text{g/ml}$. Interestingly, disruption of *UPC2A* led to not only a reduction in the fluconazole MIC from 8 $\mu\text{g/ml}$ to 0.5 $\mu\text{g/ml}$ but also a reduction in the fluconazole MFC from >64 $\mu\text{g/ml}$ to 1 $\mu\text{g/ml}$ in the SDD isolate. Similar reductions in MIC were observed for other azoles and the SBIs terbinafine and lovastatin, but not for amphotericin B or the echinocandin anidulafungin, which act directly on ergosterol in the cell membrane or on the cell wall, respectively. Despite the reduction in ergosterol content, we expected no change in amphotericin B susceptibility based on previous findings for *C. albicans* and *C. glabrata* (24, 29, 63). We agree with the hypothesis of Silver et al. that this could potentially be due to the amount of remaining ergosterol being

above the threshold necessary for amphotericin B to cause cell death (29).

As fluconazole is the most commonly prescribed azole antifungal, we examined its activity against these strains more closely. We used a 72-h endpoint in a broth microdilution assay to assess the ability of the organism to resume growth in the presence of fluconazole. Disruption of *UPC2A* greatly impeded the ability of isolate SM1 to resume growth in even relatively low concentrations of the drug. Likewise, when spotted on YPD plates containing either 5 or 10 $\mu\text{g/ml}$ of fluconazole, isolate SM1 was unable to grow when *UPC2A* was disrupted. Similar results were observed by time-kill analysis in RPMI medium, in which isolate SM1 exhibited modest growth at 24 h in the presence of 10 $\mu\text{g/ml}$ of fluconazole, whereas growth was inhibited in the absence of *UPC2A*. These results further demonstrate that *UPC2A* is required for optimal azole activity against *C. glabrata*.

We then examined the azole-resistant clinical isolate SM3 under the same conditions and observed remarkably similar results. Disruption of *UPC2A* led to increased susceptibility to fluconazole, resulting in an MIC equal to that of SDD isolate SM1. Moreover, the MFC for fluconazole was reduced from >256 $\mu\text{g/ml}$ to 16 $\mu\text{g/ml}$. Disruption of *UPC2A* resulted in similar effects on susceptibility to other azoles and sterol biosynthesis inhibitors. Growth in the presence of fluconazole was likewise found to be greatly reduced when measured at 72 h in liquid RPMI medium, on YPD plates containing 10 $\mu\text{g/ml}$ of fluconazole and by time-kill analysis at 24 h in 10 $\mu\text{g/ml}$ of fluconazole. These results demonstrate that *UPC2A* is required for high-level azole resistance in *C. glabrata*.

One possible explanation for the increased fluconazole susceptibility observed in these isolates is a change in expression of the

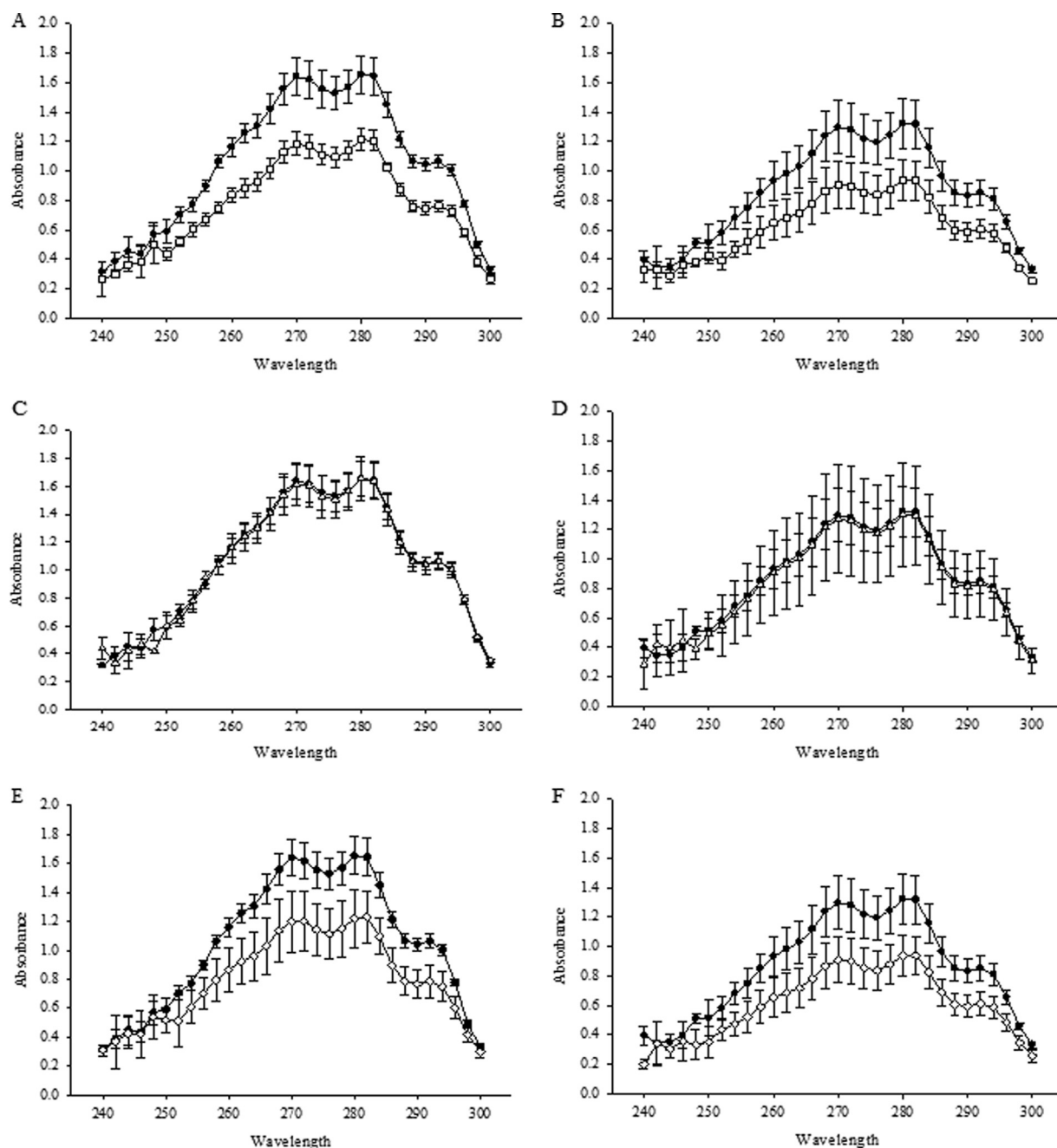


FIG 5 Ergosterol quantification for *UPC2A* disruptants in an SDD clinical isolate, SM1 (A, C, and E), and matched resistant isolate SM3 (B, D, and F). Genotypes studied were as follows: wild-type *UPC2A* allele (solid circles), $\Delta upc2a$ (open squares) (A and B), $\Delta upc2b$ (open triangles) (C and D), and $\Delta upc2a \Delta upc2b$ (open diamonds) (E and F). The results for the *UPC2A* disruptants shown in panels A, B, E, and F showed statistically significantly lower ergosterol content than was found in the wild type ($P < 0.05$). Knockout strains with the wild-type allele complemented back in showed no difference in ergosterol content compared to those of the wild-type isolates (data not shown). Strains were grown in RPMI medium for 16 h, followed by a heptane extraction and spectrophotometric scan between 240 and 300 nm (41). Each isolate was extracted and analyzed in three independent experiments.

genes encoding the multidrug resistance transporters Cdr1, Pdh1, and Snq2. Indeed, it has been shown that in *C. albicans*, Upc2 binds the promoter of *CDR1*, suggesting that it may contribute to its regulation (30). Interestingly, the sterol response element recognized by Upc2, as defined by Znaidi et al., is present in the promoter region of *CDR1* in *C. glabrata* as well (30). It has also been shown that fluconazole treatment activates the transcription factor Pdr1 and induces the expression of its targets, multidrug

transporter genes (64). We therefore examined the influence of *UPC2A* on both baseline and inducible expression of *PDR1*, *CDR1*, *PDH1*, and *SNQ2* in isolates SM1 and SM3. Disruption of *UPC2A* had no effect on baseline expression of these genes in isolate SM1 or on their constitutive overexpression in isolate SM3. Interestingly, disruption of *UPC2A* reduced the inducible expression of all four genes in response to fluconazole. This suggests that Upc2A is required for optimal activation of the Pdr1 transcrip-

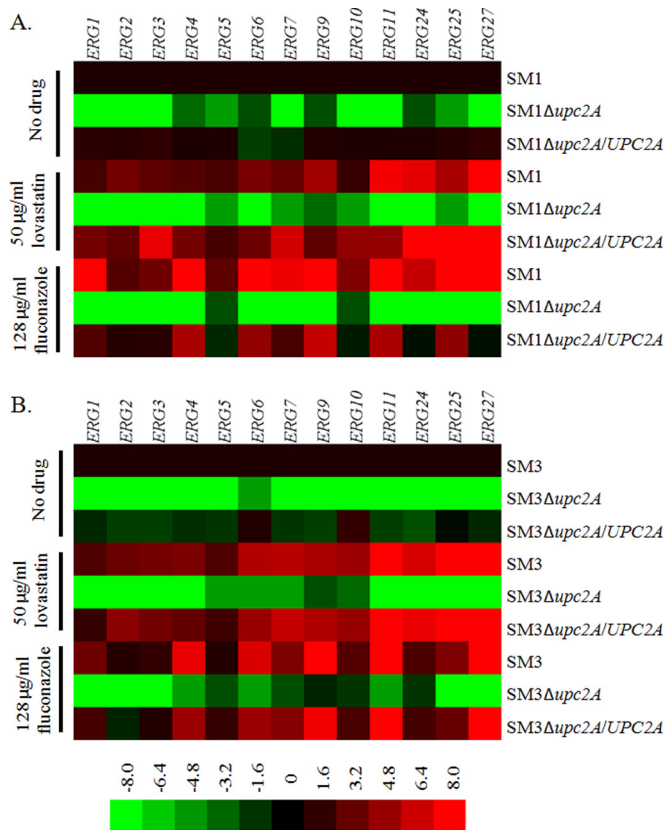


FIG 6 Effect of *UPC2A* disruption on both baseline and sterol biosynthesis inhibitor-induced expression of ergosterol biosynthesis genes. RNA was isolated from mid-log-phase cultures in parent clinical isolates, $\Delta upc2A$ mutants, and revertants. qRT-PCR analysis of relative gene expression was performed. (A) SM1, $\Delta upc2A$ mutant, and revertant gene expression. Gene expression was normalized to that in the untreated SM1 sample for each ergosterol biosynthesis gene. (B) SM3, $\Delta upc2A$ mutant, and revertant gene expression. Gene expression was normalized to that in the untreated SM3 sample for each ergosterol biosynthesis gene.

tional pathway. The diminished capacity of isolate SM1 to respond to fluconazole stress by upregulating these transporter genes may contribute to its increased susceptibility to the azoles and other sterol biosynthesis inhibitors.

Another possible explanation for the increased fluconazole susceptibility observed in these isolates is a change in the expression of genes involved in ergosterol biosynthesis that are regulated by *UPC2A*. Both SM1 and SM3 exhibited reduced cellular ergosterol when *UPC2A* was disrupted. Likewise, baseline expression as well as inducible expression by fluconazole or lovastatin of all ergosterol biosynthesis genes analyzed was reduced in the absence of *UPC2A*. Importantly, the azole target, *ERG11*, is included in this group of ergosterol biosynthesis genes, which would result in a reduced amount of target that would need to be inhibited in order to effectively inhibit ergosterol biosynthesis. These data suggest that the global impact on sterol biosynthesis in the absence of *UPC2A* greatly increases the vulnerability of *C. glabrata* to fluconazole, even in the setting of Pdr1 activation and overexpression of *CDR1*, *PDH1*, and *SNQ2*.

Additionally, the incorporation of alternate sterols into the cell membrane affects fluidity of the membrane and may compromise the ability of the efflux pumps to function properly. Krishnamur-

thy and Prasad demonstrated altered accumulation of various compounds when tested against a panel of *S. cerevisiae* *ERG* mutants expressing *C. albicans* *Cdr1* (65). Altered membrane fluidity and asymmetry have also been associated with increased resistance to fluconazole in *C. albicans* independent of efflux pump function (66). Taken together, our findings suggest that *Upc2A* and the transcriptional activation pathway it regulates represent potential targets for overcoming azole antifungal resistance in *C. glabrata*.

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