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UPC2 Is Universally Essential for Azole Antifungal Resistance in *Candida albicans*

Erin M. Vasicek,[a] Elizabeth L. Berkow,[a] Stephanie A. Flowers,[a] Katherine S. Barker,[a] P. David Rogers[a,b,c]

Department of Clinical Pharmacy, College of Pharmacy,[a] and Department of Pediatrics, College of Medicine,[b] University of Tennessee Health Science Center, and Children's Foundation Research Center at Le Bonheur Children's Medical Center,[c] Memphis, Tennessee, USA

In *Candida albicans*, the transcription factor Upc2 is central to the regulation of ergosterol biosynthesis. *UPC2*-activating mutations contribute to azole resistance, whereas disruption increases azole susceptibility. In the present study, we investigated the relationship of *UPC2* to fluconazole susceptibility, particularly in azole-resistant strains. In addition to the reduced fluconazole MIC previously observed with *UPC2* disruption, we observed a lower minimum fungicidal concentration (MFC) for a *upc2*Δ/Δ mutant than for its azole-susceptible parent, SC5314. Moreover, the *upc2*Δ/Δ mutant was unable to grow on a solid medium containing 10 μg/ml fluconazole and exhibited increased susceptibility and a clear zone of inhibition by Etest. Time-kill analysis showed higher fungistatic activity against the *upc2*Δ/Δ mutant than against SC5314. *UPC2* disruption in strains carrying specific resistance mutations also resulted in reduced MICs and MFCs. *UPC2* disruption in a highly azole resistant clinical isolate containing multiple resistance mechanisms likewise resulted in a reduced MIC and MFC. This mutant was unable to grow on a solid medium containing 10 μg/ml fluconazole and exhibited increased susceptibility and a clear zone of inhibition by Etest. Time-kill analysis showed increased fungistatic activity against the *upc2*Δ/Δ mutant in the resistant background. Microarray analysis showed attenuated induction by fluconazole of genes involved in sterol biosynthesis, iron transport, or iron homeostasis in the absence of *UPC2*. Taken together, these data demonstrate that the *UPC2* transcriptional network is universally essential for azole resistance in *C. albicans* and represents an attractive target for enhancing azole antifungal activity.

The increased frequency of invasive fungal infections in recent decades is directly related to an expansion in the immunocompromised patient population, including those with HIV/AIDS, cancer chemotherapy patients, neutropenic patients, recipients of transplants with indwelling catheters, and patients receiving antibiotics (1–3). *Candida* species collectively are the fourth leading cause of nosocomial infections in the United States, and such infections are associated with unacceptably high rates of mortality (2, 4–6). *Candida albicans* is the most prevalent opportunistic human fungal pathogen and causes a wide variety of infections, from superficial mucosal infections to invasive disseminated disease, despite the availability of effective antifungal treatment. Moreover, the most common opportunistic infection among AIDS patients is oropharyngeal candidiasis (OPC), and chronic cases continue despite the availability of highly active antiretroviral therapy (HAART), due to poor compliance, lack of access, and failure of HAART (7–11).

The azoles, particularly fluconazole (FLC), are the most widely used class of antifungals for the treatment of *Candida* infections (2, 12). Fluconazole acts by inhibiting the protein product of *ERG11*, lanosterol 14α-demethylase, which leads to ergosterol depletion and the accumulation of toxic methylated sterol precursors, resulting in the inhibition of growth (13). In an immunocompromised host, the fungistatic nature of fluconazole limits its efficacy against *Candida* species (14, 15). *C. albicans* exhibits inhibited growth in the presence of this fungistatic drug, and it is generally believed that such fungistatic activity facilitates the development of resistance, creating problems for treatment, especially in immunocompromised patients with OPC (16, 17). Currently known mechanisms of resistance to the azoles include alterations in the expression of drug efflux pump (*CDR1*, *CDR2*, and *MDR1*) and ergosterol biosynthesis (*ERG*) genes and mutations in *ERG11* (18). These mechanisms are frequently combined, resulting in a stepwise development of resistance over time.

In *C. albicans*, *UPC2* encodes a zinc cluster transcription factor that regulates the expression of genes involved in ergosterol biosynthesis, including *ERG11* (19, 20). Disruption of *ERG11* or pharmacologic inhibition of the enzyme it encodes leads to reduced ergosterol production and the accumulation of alternate sterols, including lanosterol, eburicol, obtusifoliol, 14α-methyl fecosterol, and 14α-methylergosta-8,24(28)-dien-3β,6α-diol (21). Biosynthesis of these alternate sterols does not require *ERG11* but does require other genes in the ergosterol biosynthesis pathway, some of which appear to be regulated by Upc2. We reasoned that since Upc2 is required for the transcriptional activation of other genes of the sterol biosynthesis pathway, the disruption of *UPC2* might result in enhanced activity of the azole antifungals in azole-resistant as well as azole-susceptible isolates. In the present study, we further examined the role of *UPC2* in azole antifungal activity against both azole-susceptible and azole-resistant strains of *C. albicans*, in particular an azole-resistant clinical isolate and mutant strains that represent four of the major azole resistance mechanisms. Taken together, our results indicate that the *UPC2* transcriptional network is universally essential for fluconazole resistance in *C. albicans*.

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Address correspondence to P. David Rogers, progers3@uthsc.edu.

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TABLE 1 *C. albicans* strains used in this study

Strain	Designation	Strain background[a]	Relevant characteristic(s) or genotype[b]	Source or reference
SC5314	SC5314	N/A	*UPC2-1*/*UPC2-2*	ATCC
Clinical isolate 2-79	2-79	N/A	Susceptible isolate	53
Clinical isolate 12-99	12-99	N/A	Resistant isolate	53
UPC2M4A	*upc2*Δ/Δ strain	SC5314	*upc2-1*Δ::FRT/*upc2-2*Δ::FRT	25
UPC2M2K21A	*upc2*Δ/Δ+*UPC2* strain	UPC2M2A	*upc2-1*Δ::FRT/*UPC2*$^{S1\text{-}1}$-*caSAT1*	25
12-99UPC2A5C1A	12-99*upc2*Δ/Δ	12-99	*upc2*Δ::FRT/*upc2*Δ::FRT	This study
12-99UPC2A5C1A4A	12-99*upc2*Δ/Δ+*UPC2*	12-99UPC2A5C1A	*upc2*Δ::FRT/*UPC2*$^{12\text{-}99}$-*caSAT1*	This study
10C1B1M1	*ERG11*^{K143R} mutant	SC5314	*ERG11*^{K143R}::FRT/*ERG11*^{K143R}::FRT *UPC2-1*/*UPC2-2*	This study
10C1B1M1UPC2C9H	*ERG11upc2*Δ/Δ mutant	10C1B1M1	*ERG11*^{K143R}::FRT/*ERG11*^{K143R}::FRT *upc2-1*Δ::FRT/*upc2-2*Δ::FRT	This study
SCMRR1R34A	*MRR1*^{P683S} mutant	SC5314	*MRR1*^{P683S}::FRT/*MRR1*^{P683S}::FRT *UPC2-1*/*UPC2-2*	20
Δ*upc2*MRR1R34A	*MRR1upc2*Δ/Δ mutant	SCMRR1R34A	*MRR1*^{P683S}::FRT/*MRR1*^{P683S}::FRT *upc2-1*Δ::FRT/*upc2-2*Δ::FRT	20
SCTAC1R34A	*TAC1*^{G980E} mutant	SC5314	*TAC1*^{G980E}::FRT/*TAC1*^{G980E}::FRT *UPC2-1*/*UPC2-2*	32
SCupc2TAC1R34A1A14A	*TAC1upc2*Δ/Δ mutant	SCTAC1R34A	*TAC1*^{G980E}::FRT/*TAC1*^{G980E}::FRT *upc2-1*Δ::FRT/*upc2-2*Δ::FRT	This study

[a] N/A, not applicable.

[b] FRT, FLP recombination target.

MATERIALS AND METHODS

Strains and growth conditions. All *C. albicans* strains (Table 1) were stored as frozen stocks in 40% glycerol at −80°C. YPD (1% yeast extract, 2% peptone, and 1% dextrose) agar plates and YPD liquid medium were used for routine growth of strains at 30°C. Iron-poor agar plates and medium were prepared by adding 200 μM bathophenanthroline disulfonic acid (BPS) to YPD. Iron-rich agar plates and medium were prepared by adding 100 μM iron chloride ($FeCl_3$) to YPD. For CFU counts during time-kill analysis, PDA (0.4% potato starch, 2% dextrose, and 1.5% agar) plates were used, and cultures grown on PDA were incubated at 35°C.

Drug susceptibility testing. The MICs of fluconazole (FLC) were determined by using broth microdilution as described by NCCLS (now CLSI) standard M27-A2 (22), modified by using YPD medium (iron replete), iron-poor medium, or iron-rich medium, and were read both visually and spectrophotometrically at 24, 48, and 72 h. Minimum fungicidal concentrations (MFCs) were measured by removing 2 μl from each well of the MIC plate and plating onto YPD agar. Also, serial dilutions from a suspension with an optical density at 600 nm (OD_{600}) of 0.1 were diluted 4-fold, and 2 μl of each dilution was plated onto YPD agar plates or iron-poor agar plates with or without 10 μg/ml fluconazole and was then incubated at 30°C for 24 and 48 h. Fluconazole activity was also assessed by Epsilometer test strips (Etest strips) (bioMérieux) according to the manufacturer's instructions with the following modifications. A standardized cell suspension (a 0.5 McFarland standard) was used to create a confluent lawn across YPD agar plates or iron-poor agar plates prior to Etest strip placement, and the cells were then incubated at 30°C for 24 and 48 h. Time-kill analyses were performed with a cell suspension at a 0.5 McFarland standard, which was 10-fold diluted into YPD medium with or without 10 μg/ml fluconazole and was incubated at 35°C. Aliquots were removed at 0, 6, 12, and 24 h, 10-fold serially diluted, and plated onto PDA. CFU were counted in duplicate after 48 h at 35°C and were plotted on a log-scale curve versus time (23).

Construction of strains with *ERG11* mutant alleles. *Candida albicans ERG11* (*CaERG11*) coding sequences were amplified by PCR (*Pfu* DNA polymerase; Stratagene) from *C. albicans* genomic DNA using primers *ERG11*-A and *ERG11*-E. Products were cloned into pCR-BLUNTII-TOPO using a Zero Blunt TOPO PCR cloning kit (Invitrogen) and were transferred into *Escherichia coli* TOP10 cells with selection on LB agar plates containing 50 μg/ml kanamycin. Plasmid DNA was purified (QIAprep; Qiagen, Germantown, MD) and was sequenced on an ABI model 3130XL genetic analyzer using the *ERG11* sequencing primers (Table 2), resulting in full-length sequence from both strands of the *CaERG11* gene. The sequencing was performed using six sets of clones derived from three independent PCRs for each strain/isolate sequenced.

Sequenced plasmids of the *ERG11* open reading frame (ORF) whose predicted translation indicated an amino acid substitution were digested with restriction enzymes ApaI and XhoI, which excised the full-length ORF from the plasmid, and the *ERG11* alleles were cloned upstream of the *SAT1*-flipper cassette into the ApaI and XhoI sites of plasmid pSFS2 (24). The *ERG11* downstream segments were amplified with *Ex Taq* polymerase (TaKaRa) using primers *ERG11*-C and *ERG11*-D and were cloned downstream of the *SAT1*-flipper cassette in pSFS2 using the NotI and SacII sites.

Construction of *UPC2* deletion strains. Plasmid pSFS2 contains the entire *SAT1*-flipper cassette (24). The *SAT1*-flipper cassette consists of the *SAT1* selectable marker, which confers resistance to nourseothricin, and the *FLP* flipper recombinase gene, both flanked by *FRT* sites (flipper recombinase target sequences). The *UPC2* deletion cassette (pUPC2M2), where the 5′ upstream sequence from position −373 to +15 was cloned downstream of the *SAT1*-flipper cassette while the 3′ sequence from position +2097 to +2437 was cloned upstream of the *SAT1*-flipper cassette, was developed by Dunkel et al. (25) (Table 1). Upon transformation of the parent strain with the gel-purified SacI-ApaI fragment from pUPC2M2, the *SAT1*-flipper cassette was inserted into the coding region of one allele from position +16 to +2096, and such positive transformants (Nou^R) were selected on YPD-nourseothricin agar plates containing 200 μg/ml of nourseothricin. The *FLP* gene was induced by growing the transformants in YPD medium for 24 h without selective pressure. Positive cells (Nou^S) were selected by replica plating onto YPD plates with or without 200 μg/ml of nourseothricin. Upon induction of the *FLP* gene, the cassette was excised such that only one copy of the *FRT* site remained in the locus. Another round was required to disrupt the second allele. Appropriate gene disruption and complementation for two independent strains were confirmed by Southern hybridization (24).

Isolation of genomic DNA and Southern hybridization. Genomic DNA was isolated as described previously (26). Four micrograms of DNA was digested with an appropriate restriction endonuclease, separated on a 1% agarose gel, and, after staining with ethidium bromide, was transferred by vacuum blotting to a nylon membrane and fixed by UV cross-linking. Southern hybridization with enhanced-chemiluminescence-labeled probes was performed with the Amersham ECL Direct nucleic acid labeling and detection system according to the manufacturer's instructions.

TABLE 2 Primers used in this study

Primer	Sequence[a]
qRT-PCR	
ACT1-F	5′-ACGGTGAAGAAGTTGCTGCTTTAGTT-3′
ACT1-R	5′-CGTCGTCACCGGCAAAA-3′
BMR1-F	5′-ACATAAATACTTTGCCCATCCAGAA-3′
BMR1-R	5′-AAGAGTTGGTTTGTAATCGGCTAAA-3′
CDR1-F	5′-ATTCTAAGATGTCGTCGCAAGATG-3′
CDR1-R	5′-AGTTCTGGCTAAATTCGTAATGTTTTC-3′
CDR2-F	5′-TAGTCCATTCAACGGCAACATT-3′
CDR2-R	5′-CACCCAGTATTTGGCATTGAAA-3′
CFL4-F	5′-GCAATGGTTGACAGGTTGGAA-3′
CFL4-R	5′-GCAATGTGACGATGATAAGTGACAA-3′
ERG11-F	5′-CCCCTATTAATTTTGTTTTCCCTAATTTAC-3′
ERG11-R	5′-CACGTTCTCTTCTCAGTTTAATTTCTTTC-3′
FET3-F	5′-GCCGGTGTCTTAGGTTTAGCC-3′
FET3-R	5′-CTAGCAACTCTTTCTTCAACATCGG-3′
FRP1-F	5′-CTTCCAATACCATCCATTCACGAT-3′
FRP1-R	5′-ATCTCCCCACTTTCAGCAAGAC-3′
FTR1-F	5′-ATTGTTGTTTCAGTGCTTTTGGC-3′
FTR1-R	5′-GGTCGGAACTACCACCCATAGA-3′
ERG11 mutant construction	
ERG11-A	5′-GGGCCCGGGTTATTTGAGAACAGCC-3′
ERG11-B	5′-ATCCGTTCTCGAGCACTAAGGGACAA-3′
ERG11-C	5′-GTAATCAATTGAGCTCTTTTAACTTT-3′
ERG11-D	5′-GATTATAGTTCCGCGGTGGTTTTACC-3′
ERG11-E	5′-TGATGGTTTTTGTCCACTGGCTCGAG-3′
ERG11 sequencing	
ERG11 seq B	5′-TATTTTCACTGCTTCAAGATCT-3′
ERG11 seq C	5′-CCAAAAGGTCATTATGTTTTAG-3′
ERG11 seq E	5′-AATGAGGTTTTTCACCTAAATG-3′
ERG11 seq F	5′-CCCTTTACCGAAAACTGGAGTA-3′
T7	5′-TAATACGACTCACTATAGGG-3′
M13R	5′-CAGGAAACAGCTATGACC-3′
UPC2 mutant construction	
UPC2-A	5′-GGGCCCGAGATCTTGATGTCATTAG-3′
UPC2-B	5′-CTCGAGCTATATCTTCAATGAACTG-3′
UPC2-C	5′-CCGCGGACAGGTCAATACCGCGTAG-3′
UPC2-D	5′-GAGCTCGTTCCTCTAGTATCACTCTT-3′
UPC2-E	5′-CTCGAGCACCACAGTAACGAATCAC-3′

[a] Underlining reflects the introduction of a restriction site sequence.

RNA isolation for quantitative reverse transcription-PCR (qRT-PCR). RNA was isolated using a small-scale hot phenol method of RNA isolation described by Schmitt et al. (27). Briefly, overnight cultures were diluted to an OD$_{600}$ of 0.2 in 20 ml YPD and were then incubated at 30°C with shaking for 3 h. Cells were collected by centrifugation and were stored at -80°C. Cell pellets were resuspended in 950 μl of AE buffer (50 mM sodium acetate [pH 5.3], 10 mM EDTA [pH 8.0]) and were then transferred to a 2-ml RNase-free microcentrifuge tube containing 950 μl acid phenol (pH 4.3) with 1% SDS. Cells were incubated at 65°C for 10 min; then lysates were clarified by centrifugation. The supernatant was then divided into two new 2-ml microcentrifuge tubes containing 950 μl of chloroform, and the contents of each tube were mixed. The sample was then subjected to centrifugation again, and the top aqueous layer was transferred to a new tube containing 1 ml of isopropanol and 100 μl 2 M sodium acetate. The RNA pellet was subsequently washed with 500 μl of 70% ethanol and was collected by centrifugation. The RNA pellet was resuspended in DNase/RNase-free H$_2$O. Quantity and purity were determined spectrophotometrically by A_{260} and A_{280}.

Quantitative RT-PCR. First-strand cDNAs were synthesized from 1 μg of total RNA using the SuperScript first-strand synthesis system for RT-PCR (Invitrogen). Gene-specific primers (Table 2) were designed using Primer Express software (Applied Biosystems) and were synthesized by Integrated DNA Technologies (Coralville, IA). Quantitative PCRs were performed in triplicate using the 7000 sequence detection system (Applied Biosystems), independently amplifying *ACT1* (normalizing gene) and the genes of interest (GOI) as described previously (28).

RNA isolation for microarray. RNA was isolated using a large-scale version of the hot phenol method of RNA isolation described by Schmitt et al. (27). Briefly, overnight cultures were diluted to an OD$_{600}$ of 0.005 in 100 ml YPD and were then incubated at 30°C with shaking for an additional 8 h to an OD$_{600}$ of 1.0. Cultures were diluted again to an OD$_{600}$ of 0.025 in 100 ml fresh YPD, allowed to incubate at 30°C with shaking for one doubling, inoculated with or without 10 μg/ml FLC, and then incubated at 30°C with shaking for 6 h. Cells were collected by centrifugation and were stored at -80°C. Cell pellets were resuspended in 12 ml of AE buffer (50 mM sodium acetate [pH 5.3], 10 mM EDTA [pH 8.0]) and were then transferred to 50-ml Oak Ridge tubes treated with RNase Away (Molecular BioProducts) containing 12 ml acid phenol (pH 4.3) with 1% SDS. Cells were incubated at 65°C for 10 min; then lysates were clarified by centrifugation. The supernatant was then transferred to a new tube containing 15 ml of chloroform, and the contents of the tube were mixed. The sample was then subjected to centrifugation again, and the top aqueous layer was transferred to a new tube containing 1 volume of isopropanol and 0.1 volume of 2 M sodium acetate. The RNA pellet was subsequently

TABLE 3 MICs and MFCs in YPD in the SC5314 background

Medium and strain	Relevant characteristics or genotype	MIC (μg/ml)			MFC (μg/ml)		
		24 h	48 h	72 h	24 h	48 h	72 h
YPD							
SC5314	*UPC2-1*/*UPC2-2*	0.5	0.5	>64	>64	>64	>64
*upc2*Δ/Δ mutant	*upc2-1*Δ::FRT/*upc2-2*Δ::FRT	≤0.125	0.25	0.25	0.25	0.25	0.25
*upc2*Δ/Δ+*UPC2* strain	*upc2-1*Δ::FRT/$UPC2^{S1-1}$-ca*SAT1*	0.5	0.5	>64	>64	>64	>64
YPD + BPS							
SC5314	*UPC2-1*/*UPC2-2*	0.5	0.5	0.5	>64	>64	>64
*upc2*Δ/Δ mutant	*upc2-1*Δ::FRT/*upc2-2*Δ::FRT	NG[a]	NG	<0.125	0.125	0.125	<0.125
*upc2*Δ/Δ+*UPC2* strain	*upc2-1*Δ::FRT/$UPC2^{S1-1}$-ca*SAT1*	0.5	0.5	0.5	>64	>64	>64
YPD + FeCl$_3$							
SC5314	*UPC2-1*/*UPC2-2*	1	1	>64	>64	>64	>64
*upc2*Δ/Δ mutant	*upc2-1*Δ::FRT/*upc2-2*Δ::FRT	<0.125	0.25	0.5	0.25	0.25	1
*upc2*Δ/Δ+*UPC2* strain	*upc2-1*Δ::FRT/$UPC2^{S1-1}$-ca*SAT1*	1	1	>64	>64	>64	>64

[a] NG, no growth.

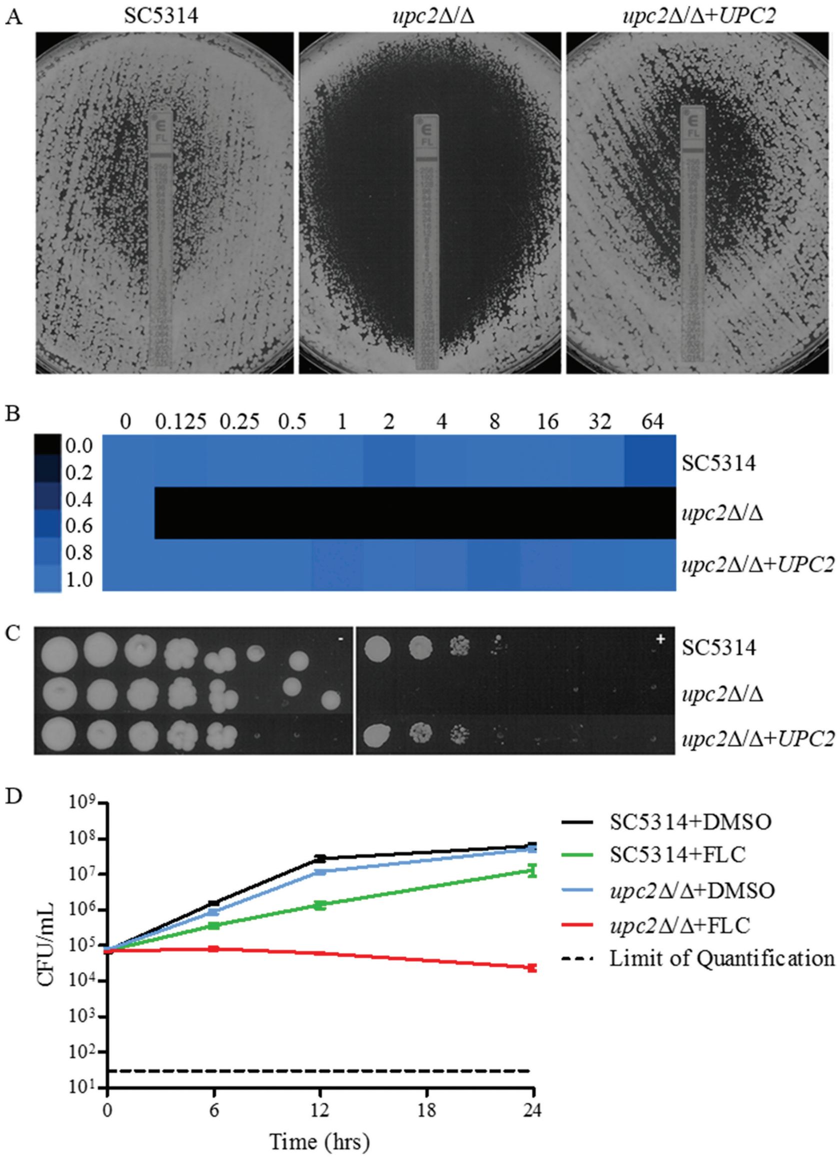

FIG 1 (A) Effects of *UPC2* on MICs and growth on YPD agar as determined by Etest. A confluent lawn of *C. albicans* was streaked prior to the addition of Etest strips and was then incubated for 48 h. (B) MIC heat map of SC5314, the *UPC2* mutant, and a complemented derivative. Susceptibility was determined by broth microdilution in YPD at 72 h (MICs in μg/ml above heat map). Growth was quantified spectrophotometrically and was assigned to a colorimetric scale. (C) Effect of *UPC2* on the ability to grow on a solid medium containing fluconazole. From 4-fold serial dilutions of *C. albicans* strains, 2-μl aliquots were spotted onto YPD agar with (right) or without (left) 10 μg/ml FLC and were incubated for 48 h. (D) Effect of fluconazole on *UPC2* in a time-kill assay. SC5314 or *upc2*Δ/Δ cells were diluted in YPD medium containing fluconazole (10 μg/ml) or the solvent dimethyl sulfoxide (DMSO). After 0, 6, 12, and 24 h, samples from each strain and medium were diluted and were plated for CFU.

washed with 10 ml of 70% ethanol and was collected by centrifugation. The RNA pellet was resuspended in DNase/RNase-free H_2O. Quantity and purity were determined spectrophotometrically by A_{260} and A_{280}.

Transcriptional profiling. Gene expression profiles were obtained by hybridizing labeled cRNAs generated from *C. albicans* total RNA onto Affymetrix *C. albicans* custom expression arrays (CAN07; 49-5241 array format) (25), which have been described previously (29). Microarray hybridization and analysis were performed as described previously (29). Genes were considered to be differentially expressed in response to the drug if their expression changed ≥1.5-fold in two independent experiments. Genes induced by FLC were considered to be *UPC2* dependent if the induction in the deletion mutant was ≥2.0-fold (50%) less than that in the wild type.

Microarray data accession number. All microarray data are available for download from the NCBI under GEO accession number GSE57929.

RESULTS

***UPC2* disruption results in enhanced fluconazole activity.** In order to further investigate the requirement of *UPC2* for susceptibility to fluconazole, we subjected the *upc2*Δ/Δ mutant derived from azole-susceptible isolate SC5314 to various azole susceptibility tests, examining both MICs and MFCs by using nutrient-rich YPD medium in order to detect strong phenotypes in an environment that promotes growth. In agreement with previous observations, disruption of *UPC2* resulted in marked reductions in flu-

TABLE 4 MICs and MFCs in YPD in the backgrounds of strains expressing resistance mechanisms

		MIC (μg/ml)			MFC (μg/ml)		
Strain	Relevant characteristics or genotype	24 h	48 h	72 h	24 h	48 h	72 h
SC5314	*UPC2-1*/*UPC2-2*	0.5	0.5	>64	>64	>64	>64
ERG11^{K143R} mutant	*ERG11*^{K143R}::FRT/*ERG11*^{K143R}::FRT *UPC2-1*/*UPC2-2*	4	8	8	8	8	32
*ERG11upc2*Δ/Δ mutant	*ERG11*^{K143R}::FRT/*ERG11*^{K143R}::FRT *upc2-1*Δ::FRT/*upc2-2*Δ::FRT	0.5	1	1	1	1	1
MRR1^{P683S} mutant	*MRR1*^{P683S}::FRT/*MRR1*^{P683S}::FRT *UPC2-1*/*UPC2-2*	16	>64	>64	>64	>64	>64
*MRR1upc2*Δ/Δ mutant	*MRR1*^{P683S}::FRT/*MRR1*^{P683S}::FRT *upc2-1*Δ::FRT/*upc2-2*Δ::FRT	2	2	4	4	4	4
TAC1^{G980E} mutant	*TAC1*^{G980E}::FRT/*TAC1*^{G980E}::FRT *UPC2-1*/*UPC2-2*	16	>64	>64	>64	>64	>64
*TAC1upc2*Δ/Δ mutant	*TAC1*^{G980E}::FRT/*TAC1*^{G980E}::FRT *upc2-1*Δ::FRT/*upc2-2*Δ::FRT	2	2	2	2	2	2

conazole MICs by broth microdilution (Table 3), Etest (Fig. 1A), 72-h regrowth (Fig. 1B), and spot assays (Fig. 1C). Interestingly, at 24 h in YPD, the fluconazole MFC was >64 μg/ml for SC5314, whereas for the *upc2*Δ/Δ mutant it was 0.25 μg/ml (Table 3). At 48 h, the MIC for SC5314, as measured by Etest, was 1.0 μg/ml, and a halo of reduced growth (but not a clear zone of inhibition) was observed up to the Etest strip, a result consistent with the fungistatic nature of fluconazole. The 48-h MIC by Etest for the

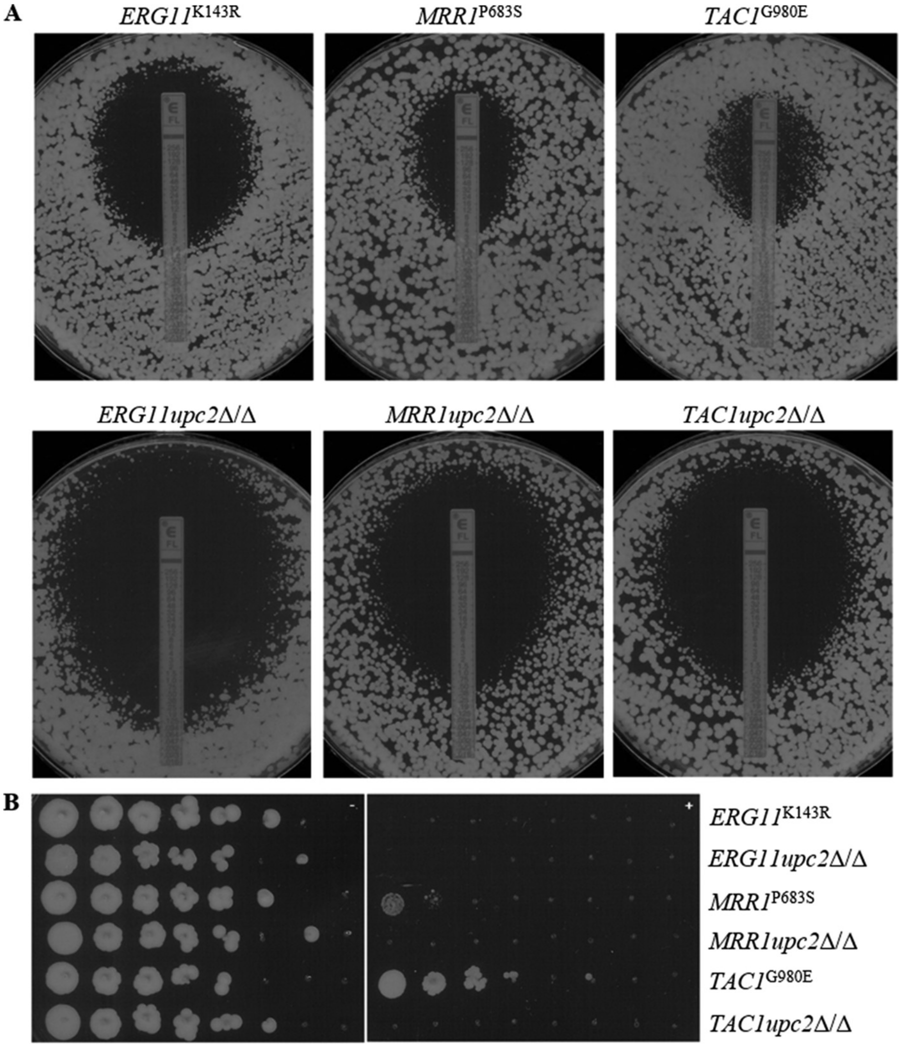

FIG 2 (A) Effects of *UPC2* in resistant backgrounds on MICs and growth on YPD agar as determined by Etest. A confluent lawn of *C. albicans* was streaked prior to the addition of Etest strips and was then incubated for 48 h. (B) Effects of *UPC2* in resistant backgrounds on the ability to grow on a solid medium containing fluconazole. From 4-fold serial dilutions of *C. albicans* strains, 2-μl aliquots were spotted onto YPD agar with (right) or without (left) 10 μg/ml FLC and were incubated for 48 h.

TABLE 5 MIC and MFCs in YPD for strains in the 12-99 background

Medium and strain	Relevant characteristics or genotype	MIC (μg/ml)			MFC (μg/ml)		
		24 h	48 h	72 h	24 h	48 h	72 h
YPD							
2-79	Susceptible isolate	1	1	>64	>64	>64	>64
12-99	Resistant isolate	>64	>64	>64	>64	>64	>64
12-99*upc2*Δ/Δ	*upc2*Δ::FRT/*upc2*Δ::FRT	4	4	4	4	4	4
12-99*upc2*Δ/Δ+*UPC2*	*upc2*Δ::FRT/*UPC2*-ca*SAT1*	64	>64	>64	>64	>64	>64
YPD + BPS							
2-79	Susceptible isolate	2	2	4	>64	>64	>64
12-99	Resistant isolate	32	32	32	>64	>64	>64
12-99*upc2*Δ/Δ	*upc2*Δ::FRT/*upc2*Δ::FRT	NG[a]	NG	NG	NG	NG	NG
12-99*upc2*Δ/Δ+*UPC2*	*upc2*Δ::FRT/*UPC2*-ca*SAT1*	32	32	32	>64	>64	>64
YPD + $FeCl_3$							
2-79	Susceptible isolate	2	>64	>64	>64	>64	>64
12-99	Resistant isolate	>64	>64	>64	>64	>64	>64
12-99*upc2*Δ/Δ	*upc2*Δ::FRT/*upc2*Δ::FRT	4	4	8	8	8	16
12-99*upc2*Δ/Δ+*UPC2*	*upc2*Δ::FRT/*UPC2*-ca*SAT1*	64	>64	>64	>64	>64	>64

[a] NG, no growth.

*upc2*Δ/Δ mutant was 0.032 μg/ml, and a clear zone of inhibition around the Etest strip was observed (Fig. 1A). We also used a 72-h endpoint for a broth microdilution assay in YPD as a way to assess the ability of the organism to resume growth in the presence of fluconazole. SC5314 was able to resume growth in fluconazole at all concentrations tested, whereas the *upc2*Δ/Δ mutant was not (Table 3; Fig. 1B). When cells were plated on YPD agar plates containing 10 μg/ml fluconazole, the level of growth in the presence of fluconazole was lower for the *upc2*Δ/Δ mutant than for SC5314 (Fig. 1C). Time-kill analysis showed higher fungistatic activity of 10 μg/ml fluconazole against the *upc2*Δ/Δ mutant than against its parent strain (Fig. 1D). All phenotypes reverted with reintegration of one allele of the disrupted gene.

***UPC2* disruption in strains containing resistance mutations in *MRR1*, *TAC1*, or *ERG11* also enhances fluconazole activity.** In order to investigate the requirement for *UPC2* in the setting of specific mechanisms of azole resistance, independent mutants were constructed in strains containing two copies of a gene conferring reduced susceptibility to fluconazole: the *MRR1*^{P683S}, *TAC1*^{G980E}, or *ERG11*^{K143R} gene. The *MRR1*^{P683S} and *TAC1*^{G980E} alleles contain gain-of-function mutations that render the transcription factors they encode constitutively active, resulting in the upregulation of either *MDR1* (*MRR1*^{P683S}) or *CDR1* and *CDR2* (*TAC1*^{G980E}), respectively, and decreased fluconazole susceptibility (30–33). The *ERG11*^{K143R} allele contains a point mutation postulated to be located near the azole access channel, interfering with the entry of fluconazole and thus resulting in decreased fluconazole susceptibility (34). Again, the disruption of *UPC2* in each background resulted in marked reductions in MICs and MFCs as determined by all methods, and the trend in susceptibility seen for the *MRR1upc2*Δ/Δ mutant was consistent with what we have observed previously (20). The MFCs at 24 h in YPD for the *ERG11upc2*Δ/Δ, *MRR1upc2*Δ/Δ, and *TAC1upc2*Δ/Δ mutants were reduced from 8 μg/ml, >64 μg/ml, and >64 μg/ml in their background strains to 1 μg/ml, 4 μg/ml, and 2 μg/ml, respectively (Table 4). At 48 h, the MICs for the *ERG11upc2*Δ/Δ, *MRR1upc2*Δ/Δ, and *TAC1upc2*Δ/Δ mutants by Etest were reduced from 1.5 μg/ml, 4 μg/ml, and 8 μg/ml in their background strains to 0.19 μg/ml, 0.5 μg/ml, and 0.5 μg/ml, respectively (Fig. 2A). The growth of the *MRR1upc2*Δ/Δ and *TAC1upc2*Δ/Δ strains when plated on YPD agar plates containing 10 μg/ml fluconazole was also reduced from that of their background strains (Fig. 2B). For the *ERG11*^{K143R} strain and its *upc2*Δ/Δ derivative, the results of growth experiments were consistent with the fluconazole MICs: both were unable to grow in the presence of fluconazole at this concentration.

Disruption of Upc2 overrides clinical drug resistance. Since *UPC2* disruption in strains containing one resistance mechanism resulted in enhanced fluconazole activity, we wanted to examine further the extent to which disruption of *UPC2* influences high-level azole resistance in the presence of multiple resistance mechanisms. We constructed *upc2*Δ/Δ mutant strains in the background of an azole-resistant clinical isolate (isolate 12-99) known to carry four of the most common mechanisms of azole resistance: overexpression of *CDR1* and *CDR2*, overexpression of *MDR1*, overexpression of *ERG11*, and a mutation in *ERG11* (35). As was observed for isolate SC5314 and the isogenic resistant strains, the disruption of *UPC2* resulted in marked reductions in MICs and MFCs as determined by all methods. The MFC at 24 h in YPD was >64 μg/ml for 12-99, whereas it was 4 μg/ml for 12-99*upc2*Δ/Δ (Table 5). The fluconazole MIC by Etest at 48 h was >256 μg/ml for 12-99, and confluent growth was observed, whereas a MIC of 3 μg/ml and a clear zone of inhibition were observed for 12-99*upc2*Δ/Δ (Fig. 3A). Likewise, in broth microdilution assays after 72 h, the parent strain was able to grow in the presence of fluconazole at all concentrations tested, whereas 12-99*upc2*Δ/Δ grew less well (Fig. 3B). The growth of 12-99*upc2*Δ/Δ was also reduced from that of its parent strain when they were plated on YPD agar plates containing 10 μg/ml fluconazole (Fig. 3C). As was observed in the SC5314 background, time-kill analysis also revealed an increased fungistatic effect for fluconazole at 10 μg/ml against 12-99*upc2*Δ/Δ (Fig. 3D). All phenotypes reverted with the reintegration of one allele of the disrupted gene.

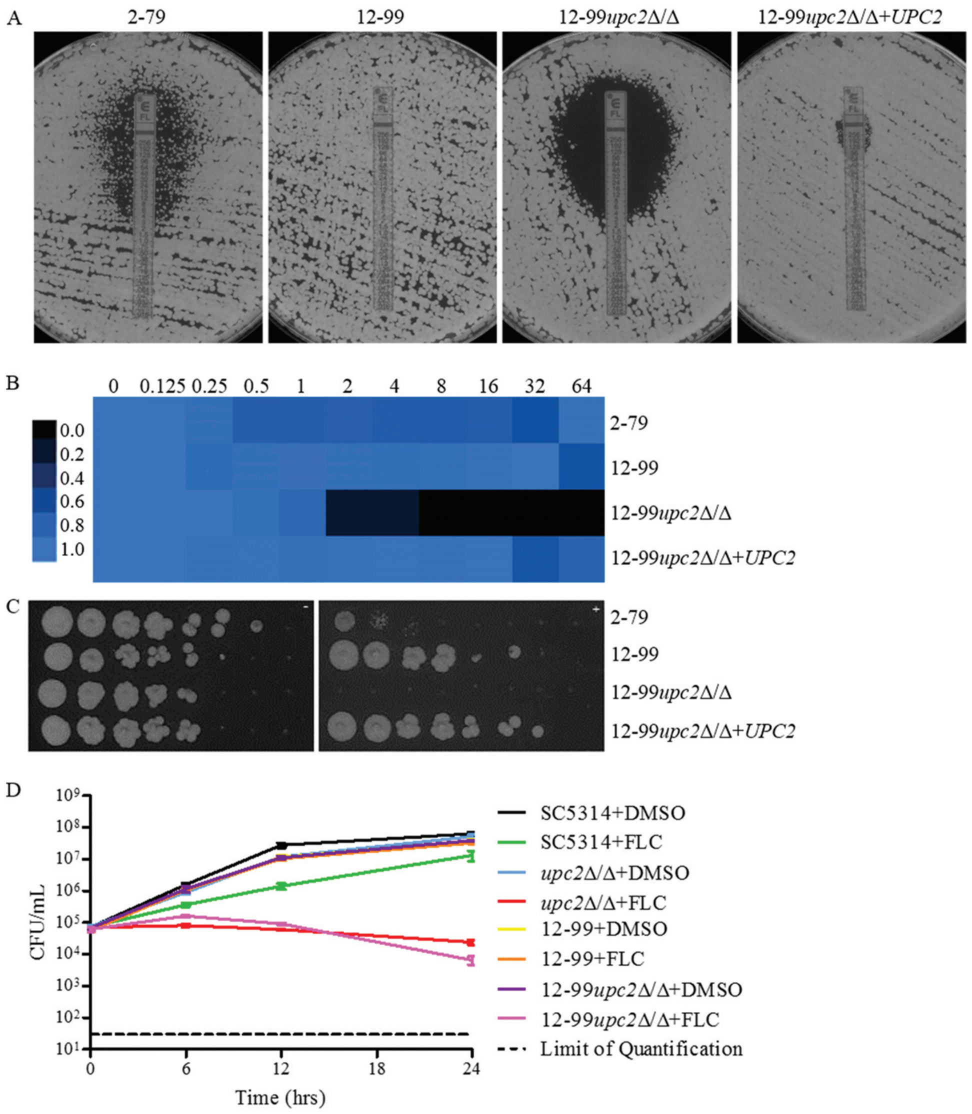

FIG 3 (A) Effects of *UPC2* in 12-99 on MICs and growth on YPD agar as determined by Etest. A confluent lawn of *C. albicans* was streaked prior to the addition of Etest strips and was then incubated for 48 h. (B) MIC heat map of 2-79, 12-99, the *UPC2* mutant, and a complemented derivative. Susceptibility was determined by broth microdilution in YPD at 72 h (MICs in μg/ml above heat map). Growth was quantified spectrophotometrically and was assigned to a colorimetric scale. (C) Effect of *UPC2* in 12-99 on the ability to grow on a solid medium containing fluconazole. From 4-fold serial dilutions of *C. albicans* strains, 2-μl aliquots were spotted onto YPD agar with (right) or without (left) 10 μg/ml FLC and were incubated for 48 h. (D) Effect of fluconazole on *UPC2* in 12-99 by a time-kill assay. 12-99 or 12-99*upc2*Δ/Δ cells were diluted in YPD medium containing fluconazole (10 μg/ml) or the solvent dimethyl sulfoxide (DMSO). After 0, 6, 12, and 24 h, samples from each strain and medium were diluted and were plated for CFU.

Expression of *ERG11*, *CDR1*, *CDR2*, and *MDR1* when *UPC2* is disrupted in resistant backgrounds. In order to determine if the enhanced fluconazole activity was due to decreased expression of *ERG11* or genes encoding efflux pumps, we measured the abundances of *ERG11*, *CDR1*, *CDR2*, and *MDR1* mRNAs by qRT-PCR in the strains containing a single resistance mechanism, clinical isolate 12-99, and their respective *upc2*Δ/Δ mutants (Fig. 4). As expected, the *upc2*Δ/Δ mutant constructed in the SC5314 background showed a reduction in baseline *ERG11* expression from that of its parent strain. This was also the case for the *upc2*Δ/Δ mutants constructed in the *ERG11*^{K143R} and 12-99 backgrounds. However, both the *TAC1*^{G980E} and *MRR1*^{P683S} strains exhibited levels of *ERG11* expression lower than that of SC5314, with no appreciable additional reduction in expression when *UPC2* was disrupted. Disruption of *UPC2* did not result in decreased expression of *CDR1*, *CDR2*, or *MDR1* in any background. Interestingly, disruption of *UPC2* in the *ERG11upc2*Δ/Δ mutant resulted in increases in the expression of these transporter genes, the significance of which is unclear. These data suggest that the enhanced activity of fluconazole observed in resistant strains lacking *UPC2* is not due to changes in transporter gene expression levels but may be associated with a reduction in the level of expression of *ERG* genes, particularly *ERG11*.

Comparison of the gene expression profiles of wild-type strain SC5314 and the *upc2*Δ/Δ mutant exposed to fluconazole. In order to identify genes whose expression in response to fluconazole is influenced by Upc2, we compared the transcriptional profiles of SC5314 and its *upc2*Δ/Δ derivative after treatment with

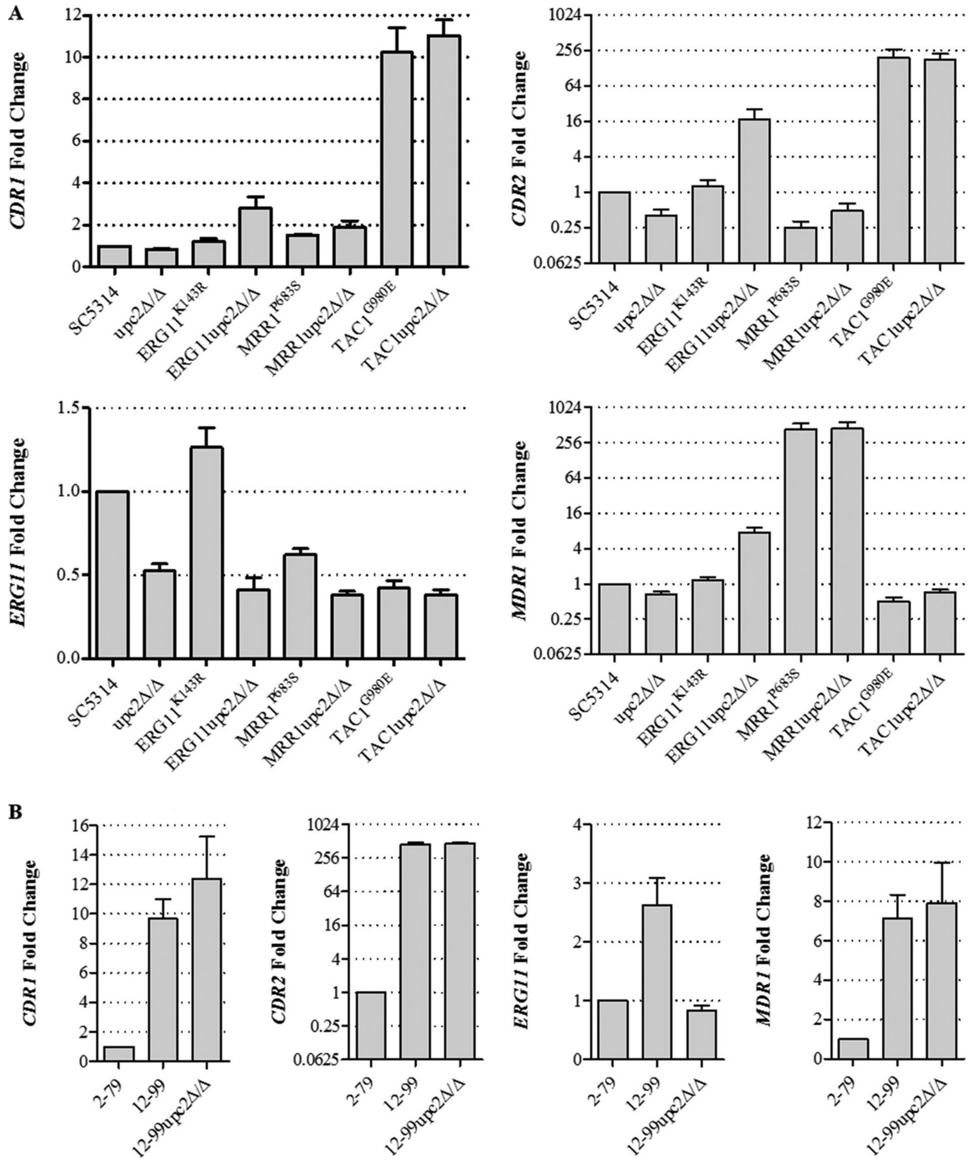

FIG 4 Expression levels of *ERG11*, *CDR1*, *CDR2*, and *MDR1*. Levels of *ERG11*, *CDR1*, *CDR2*, and *MDR1* expression in various strains were measured in triplicate by qRT-PCR and were compared to expression levels in SC5314 (A) and 2-79 (B). Error bars represent the standard errors of the means.

or without 10 μg/ml fluconazole for 6 h. Genes were considered to be differentially expressed in response to fluconazole if their expression changed by ≥1.5-fold in two independent experiments. Fluconazole-inducible genes were also considered to be *UPC2* dependent if their induction was reduced (i.e., the level of expression was ≥2.0-fold [50%] lower than that in SC5314) in the deletion mutant. By use of these criteria, there were 127 genes upregulated by fluconazole whose induction was abrogated in the absence of *UPC2* (Table 6; see also Data Set S1 in the supplemental material). The most common biological processes represented by these genes included the lipid metabolic process, iron ion transport and iron homeostasis, transport, responses to stress and chemical stimuli, and the oxidation-reduction process.

Validation of microarray data by real-time RT-PCR. In order to validate the differential expression of genes identified by the microarray, we examined the mRNA abundances for five genes of interest by using the same RNA isolated for the microarray experiments. In addition to *ERG11*, four other genes were chosen based on their involvement in iron transport and homeostasis. The correlation between the microarray data and those obtained by real-time RT-PCR was good (Fig. 5). The expression of *CFL4*, *FET3*, *FRP1*, and *FTR1* was upregulated in the wild-type strain SC5314 when treated with fluconazole but could not respond to the same extent when *UPC2* was disrupted. As expected, *ERG11* was also shown to respond to fluconazole in a *UPC2*-dependent fashion. These data suggest that the enhanced activity of fluconazole observed in both susceptible and resistant strains lacking *UPC2* may be due to dysregulation of iron homeostasis, in addition to the inability to upregulate genes involved in the ergosterol biosynthesis pathway.

Upc2 is required for growth under iron-poor conditions. In order to investigate the relationship between Upc2 and iron trans-

TABLE 6 Genes upregulated at least 1.5-fold by fluconazole that are dependent on Upc2

			Fold change in expression[b] in:				Ratio (fold change in the *upc2*Δ/Δ strain/ fold change in SC5314)	
			SC5314		*upc2*Δ/Δ strain			
Process[a]	orf19 designation	CGD name	Expt 1	Expt 2	Expt 1	Expt 2	Expt 1	Expt 2
Lipid metabolic process	orf19.1598	*ERG24*	2	1.6	0.9	0.7	0.5	0.4
	orf19.1631	*ERG6*	1.8	1.7	0.4	0.5	0.2	0.3
	orf19.2670		1.6	1.8	0.4	0.7	0.2	0.4
	orf19.3240	*ERG27*	2.8	2.4	1.4	1.1	0.5	0.4
	orf19.4982		2.1	2.3	0.7	0.8	0.3	0.4
	orf19.7585	*INO1*	9.2	20.5	0.9	0.8	0.1	0
	orf19.922	*ERG11*	1.6	1.5	0.8	0.7	0.5	0.4
Iron ion transport	orf19.1415	*FRE10*	2	4.2	0.2	0.1	0.1	0
	orf19.1932	*CFL4*	35.6	359	8	22	0.2	0.1
	orf19.4211	*FET3*	2.8	6.2	0.7	0.2	0.3	0
	orf19.4215	*FET34*	2.1	5.2	0	0.1	0	0
	orf19.5634	*FRP1*	8.9	8.2	0.1	0.2	0	0
	orf19.7219	*FTR1*	3.2	7.8	0.1	0	0	0
Iron ion homeostasis	orf19.1264	*CFL2*	2	6	0.2	0.8	0.1	0.1
	orf19.1715	*IRO1*	4.2	8.1	2.2	1.4	0.5	0.2
	orf19.5636	*RBT5*	1.7	1.6	0.2	0.2	0.1	0.2
	orf19.7114	*CSA1*	1.7	3.2	0.7	0.6	0.4	0.2
Transport	orf19.1352	*TIM22*	3.6	4.4	1.3	2	0.4	0.4
	orf19.2023	*HGT7*	20.8	21.6	9.4	7.8	0.5	0.4
	orf19.2292	*OPT4*	5.2	3.7	0	0	0	0
	orf19.2350		2.5	4.1	0.9	0.6	0.4	0.1
	orf19.2785	*ATP7*	3.1	3.1	1.3	1.5	0.4	0.5
	orf19.3026	*MAS1*	1.7	1.6	0.9	0.8	0.5	0.5
	orf19.3195	*HIP1*	1.5	2.6	0.7	0.8	0.5	0.3
	orf19.3232		24.6	5.3	1.6	1.6	0.1	0.3
	orf19.3668	*HGT2*	48.3	35.5	20.1	12.3	0.4	0.3
	orf19.3746	*IFC1*	2.3	2.7	0.2	0	0.1	0
	orf19.4335	*TNA1*	194.9	29.8	0.4	0.2	0	0
	orf19.4384	*HXT5*	70.6	75.5	16.9	9.7	0.2	0.1
	orf19.4682	*HGT17*	45	24.7	4.2	4.5	0.1	0.2
	orf19.4690		16.3	20.2	1	1.4	0.1	0.1
	orf19.5307	*JEN2*	10.3	2.7	0.4	1.3	0	0.5
	orf19.5753	*HGT10*	20.6	1.6	2.6	0.8	0.1	0.5
	orf19.6148		4.4	47.3	2	13.8	0.4	0.3
	orf19.6249	*HAK1*	5.4	5.8	1.4	1.7	0.3	0.3
	orf19.6993	*GAP2*	30.6	10.6	6	3	0.2	0.3
	orf19.7093	*HGT13*	40.9	18.8	9.8	2.6	0.2	0.1
Response to stress	orf19.1434		1.7	2.7	0.8	0.9	0.5	0.3
	orf19.3239	*CTF18*	2.7	2.4	1.4	0.8	0.5	0.4
	orf19.3672	*GAL10*	6	7.3	2.7	3.8	0.5	0.5
	orf19.4082	*DDR48*	5.8	5.7	1.9	1.3	0.3	0.2
	orf19.4093	*PES1*	2.7	3.5	0.6	1.3	0.2	0.4
	orf19.4317	*GRE3*	1.5	1.5	0.3	0.7	0.2	0.5
	orf19.496		2.6	2.3	1.1	0.6	0.4	0.3
	orf19.5902	*RAS2*	7.6	6.2	2.3	1.5	0.3	0.2
	orf19.7221	*SET3*	4.6	2.9	2.1	1.4	0.5	0.5
	orf19.921	*HMS1*	2.6	2.8	0.8	1.3	0.3	0.5
Response to chemical stimulus	orf19.4645	*BEM1*	1.5	1.9	0.4	0.6	0.3	0.3
	orf19.5591	*ADO1*	2.4	2.9	0.7	0.8	0.3	0.3
	orf19.6102	*RCA1*	2.5	3.4	1.1	1.4	0.5	0.4
	orf19.7374	*CTA4*	1.7	2.2	0.8	1	0.5	0.5
Oxidation-reduction process	orf19.1411		2.7	4.8	1.4	1.1	0.5	0.2
	orf19.1710	*ALI1*	2	1.9	0.7	0.8	0.4	0.4
	orf19.1940		1.7	1.5	0.4	0.7	0.2	0.5
	orf19.2091		2.2	2.3	0.8	0.9	0.4	0.4
	orf19.2108	*SOD6*	7.9	10.6	1.1	1.6	0.1	0.1
	orf19.4274	*PUT1*	6.8	8.8	2.5	0.6	0.4	0.1
	orf19.4747	*HEM14*	1.7	2	0.1	0.2	0	0.1

[a] Descriptions are from the *Candida* Genome Database (CGD) (http://www.candidagenome.org).

[b] Given as the ratio of expression in the presence of FLC to expression in the absence of FLC.

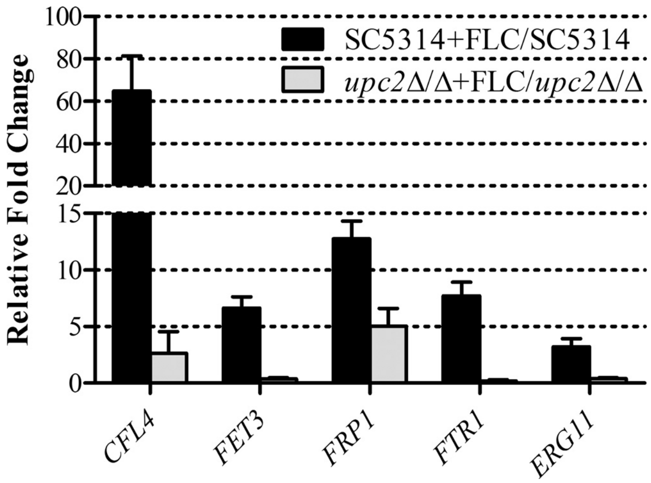

FIG 5 Validation of fluconazole-inducible and Upc2-dependent iron gene expression. Levels of *CFL4*, *FET3*, *FRP1*, *FTR1*, and *ERG11* expression were measured in triplicate by qRT-PCR and were compared to the expression levels in SC5314. Shown are the relative *n*-fold changes in gene expression in SC5314 and *upc2*Δ/Δ cells treated with fluconazole (FLC). Error bars represent the standard errors of the means.

port and homeostasis, and to examine the impact of iron on susceptibility to fluconazole, we examined the growth and fluconazole susceptibilities of SC5314, 12-99, and their respective *upc2*Δ/Δ derivatives in media with varying concentrations of iron. In broth microdilution assays using iron-replete medium (YPD only), isolates SC5314 and 12-99 were able to resume growth in the presence of all concentrations of fluconazole tested after 72 h, whereas in iron-poor medium (YPD plus BPS), these strains were unable to grow at concentrations exceeding their 24-h MICs (Tables 3 and 5). The MICs at all time points were 0.5 µg/ml for SC5314 and 32 µg/ml for 12-99. No change in MFC was observed for these strains at any time point. Meanwhile, both *upc2*Δ/Δ mutants showed little to no growth at all time points based on both MICs and MFCs. In an iron-poor medium, the fluconazole MIC for SC5314 at 48 h by Etest was 0.38 µg/ml, compared to 1 µg/ml in an iron-replete medium (Fig. 1A); however, a clear zone of inhibition was observed (Fig. 6A). A MIC of <0.016 µg/ml and a clear zone of inhibition were observed for the *upc2*Δ/Δ mutant in an iron-poor medium (Fig. 6A), compared to 0.032 µg/ml and a clear zone of inhibition in an iron-replete medium (Fig. 1A). A MIC of 64 µg/ml and a small clear zone were observed for 12-99 in an iron-poor medium (Fig. 6A), compared to >256 µg/ml and confluent growth in an iron-replete medium (Fig. 3A), and a MIC of <0.016 µg/ml and a clear zone of inhibition were observed for 12-99*upc2*Δ/Δ in an iron-poor medium (Fig. 6A), compared to 1.5 µg/ml and a clear zone in an iron-replete medium (Fig. 3A). Growth was also reduced for all strains when plated on YPD agar plates containing BPS compared to YPD alone and was further reduced by 10 µg/ml of fluconazole (Fig. 6B). Only 12-99 and its complemented derivative were able to grow on YPD agar containing both BPS and 10 µg/ml fluconazole. Importantly, disruption of *UPC2* in both SC5314 and 12-99 precluded the growth of either strain under iron-poor conditions. Conversely, high-iron conditions (YPD plus $FeCl_3$) only very modestly enhanced the abilities of these isolates to grow in the presence of fluconazole (Tables 3 and 5). These data indicate that *UPC2* is required for growth under iron-poor conditions.

DISCUSSION

Identifying novel drug targets that improve the efficacy of fluconazole is important in order to develop new therapeutic strategies to preserve the azole class of antifungals and overcome azole resistance. *UPC2* has been well characterized with regard to its impact on fluconazole susceptibility and its role in regulating genes of the ergosterol biosynthesis pathway (19, 29, 36, 37). Silver et al. and MacPherson et al. identified Upc2p as the key regulator of ergosterol metabolism in *C. albicans*, showing that azole-inducible expression of *ERG2*, *ERG7*, *ERG11*, and *ERG25* is diminished in the absence of *UPC2* (19, 36). We and others have established that in some azole-resistant isolates, specific mutations render *UPC2* constitutively active, resulting in increases in the expression of *ERG* genes (including *ERG11*), cellular ergosterol levels, and fluconazole resistance (25, 28, 29, 38–41). Moreover, *UPC2* disruption results in a reduction in cellular ergosterol content (28). This suggests that *UPC2* influences azole susceptibility through the regulation of this pathway.

In the present study, we observed that *UPC2* disruption resulted not only in enhanced fluconazole susceptibility as measured by MICs but also in a substantial reduction in fluconazole MFCs at 24, 48, and 72 h. Indeed, *UPC2* disruption in an azole-susceptible strain prevented its regrowth in YPD medium in the presence of high fluconazole concentrations after 72 h, resulted in a clear zone of inhibition around a fluconazole Etest strip, and prevented growth on a solid medium containing a therapeutically relevant concentration of fluconazole (10 µg/ml). Time-kill analysis also demonstrated a greater effect of 10 µg/ml fluconazole against the *upc2*Δ/Δ mutant than against its parent strain. Taken together, these data underscore the contribution of the Upc2 transcriptional activation pathway to azole susceptibility.

We then wanted to determine if disruption of *UPC2* might have similar effects on fluconazole-resistant isolates. For this purpose, we chose isogenic strains containing resistance mutations in *ERG11*, *MRR1*, or *TAC1*. For the strain containing the *ERG11*^{K143R} mutation, the fluconazole MIC was 8 µg/ml at 48 and 72 h in YPD. Accordingly, this strain was unable to grow in the presence of 10 µg/ml fluconazole. Although this background was not as highly resistant as others, its respective *UPC2* deletion mutant exhibited a marked drop in both the MIC and the MFC of fluconazole. The MICs and MFCs for the resistant *MRR1*^{P683S} and *TAC1*^{G980E} strains were >64 µg/ml at 48 and 72 h in YPD medium, and those for the respective *upc2*Δ/Δ mutants were reduced markedly at all time points. This trend in susceptibility is consistent with what has been observed previously for this *MRR1*^{P683S}upc2Δ/Δ mutant (20). However, in contrast to the halo of reduced confluent growth observed around the Etest strip with clinical isolate SC5314 (and generally observed with other *C. albicans* isolates), such a reduction in growth was not observed with these resistant strains, despite higher MICs and MFCs of >64 µg/ml. It has been shown recently that the constructed *MRR1*^{P683S} and *TAC1*^{G980E} mutants exhibit a fitness defect associated with the introduction of these specific resistance mutations, whereas clinical isolates carrying such mutations appear to have regained fitness (42). Such clinical isolates have likely evolved compensatory mutations that mitigate these fitness defects. This may explain the unusual growth pattern of these mutants when their susceptibilities are tested by Etest. Despite the absence of reduced confluent growth, the *upc2*Δ/Δ mutants in each background showed increased sus-

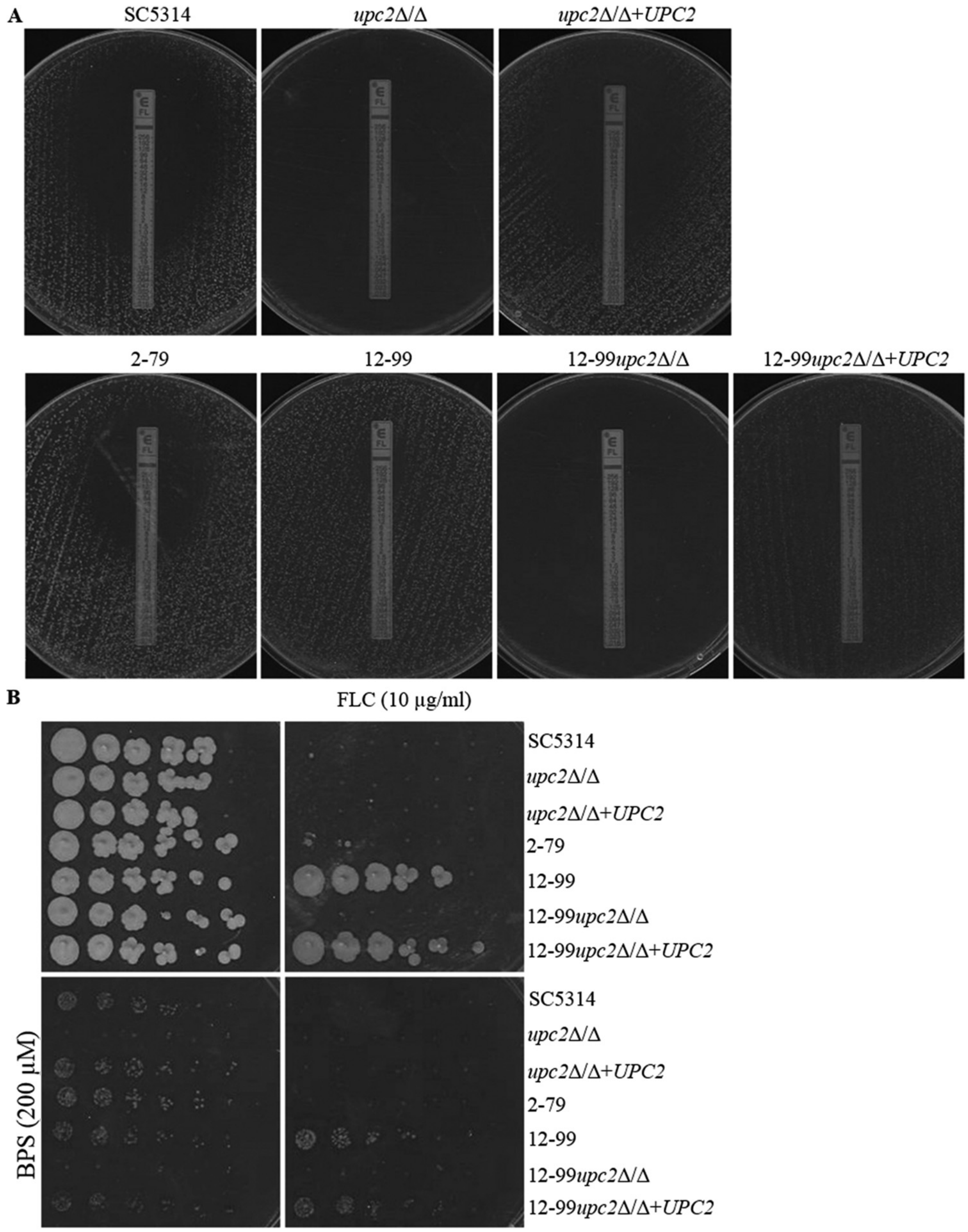

FIG 6 (A) Effect of BPS on MICs and growth on YPD agar with 200 μM BPS as determined by Etest. A confluent lawn of *C. albicans* was streaked prior to the addition of Etest strips and was then incubated for 48 h. (B) Effect of BPS on the ability to grow on a solid medium containing fluconazole. From 4-fold serial dilutions of *C. albicans* strains, 2-μl aliquots were spotted onto YPD agar with (right) or without (left) 10 μg/ml FLC and with (bottom) or without (top) 200 μM BPS and were incubated for 48 h.

ceptibility to fluconazole by Etest and an inability to grow in the presence of fluconazole at 10 μg/ml. Therefore, the loss of *UPC2* in strains containing a single mechanism of resistance also resulted in enhanced susceptibility to fluconazole, to a greater extent than that observed for their susceptible parent strain, SC5314.

The same phenotype was also observed upon disruption of *UPC2* in a highly resistant clinical isolate, 12-99, which carries all four of the most common mechanisms of resistance. 12-99*upc2*Δ/Δ exhibited a substantial reduction in fluconazole resistance from that of isolate 12-99. Although 12-99*upc2*Δ/Δ was able to resume growth in lower concentrations of fluconazole at 72 h in broth microdilution plates, it was not able to grow to the extent of 12-99 or its matched susceptible isolate, 2-79, both of which were able to grow even in the highest concentration of fluconazole. Similarly to what was observed for the *upc2*Δ/Δ mutant in the SC5314 background, fluconazole exhibited a greater fungistatic effect against 12-99*upc2*Δ/Δ than against its azole-resistant parental isolate. Indeed, the loss of *UPC2* allowed fluconazole to overcome all four mechanisms of fluconazole resistance operative in isolate 12-99 and resulted in greater fluconazole susceptibility than that seen with its matched susceptible isolate, 2-79. Moreover, the loss of *UPC2* resulted in more-pronounced effects by MFC, 72-h regrowth, and time-kill analysis than those observed for the susceptible wild-type strain, SC5314.

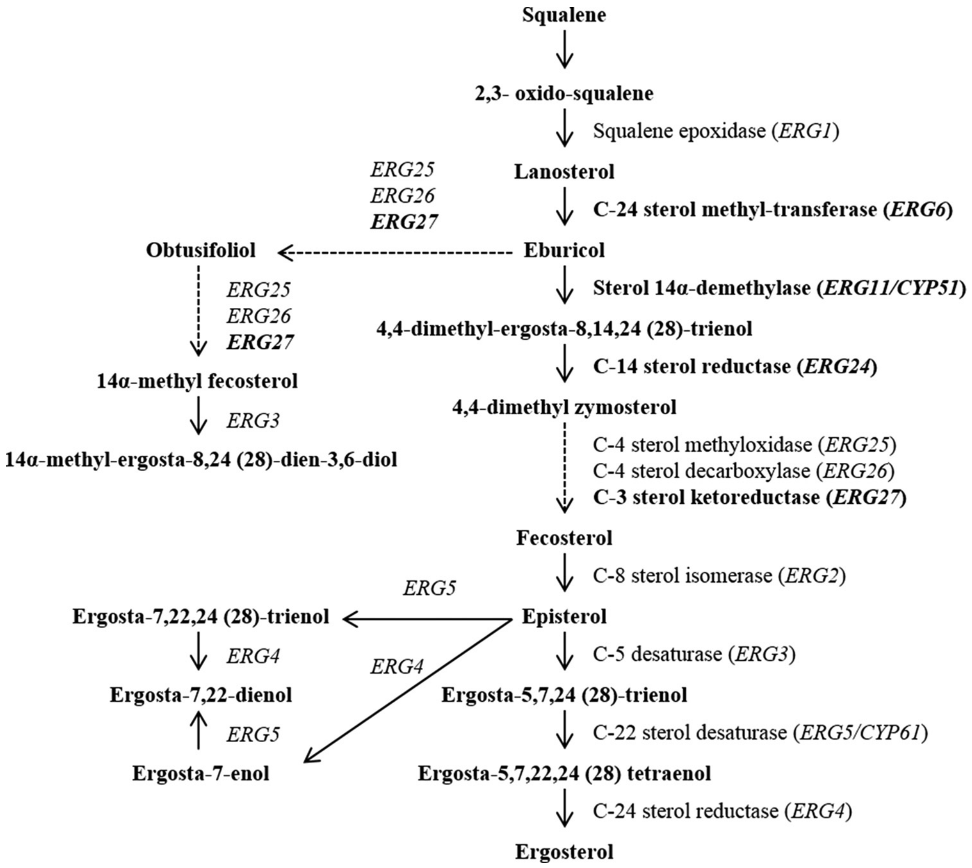

FIG 7 Sterol biosynthesis pathway in *C. albicans*. Genes whose products are shown in boldface were found to be responsive to fluconazole in a Upc2-dependent manner. Dotted lines denote multiple enzymatic steps.

One hypothesis for the way in which *UPC2* disruption overcomes drug resistance is that it influences the expression of the efflux pump genes *CDR1*, *CDR2*, and *MDR1* in these strains. Indeed, it has been shown that in *C. albicans*, Upc2 binds to the promoters of *CDR1* and *MDR1* and can regulate their expression (29). However, the expression of these genes was not affected by the absence of *UPC2* in either the constructed mutant strains or the resistant clinical isolate 12-99. The disruption of *UPC2* had such an effect on susceptibility that even with constitutive upregulation of these efflux pumps, the strains were no longer resistant to fluconazole. *CDR1* and *CDR2* remained upregulated in the *TAC1*^{G980E} strain and 12-99, as did *MDR1* in the *MRR1*^{P683S} strain and 12-99.

Another possible explanation for the increased susceptibility to fluconazole observed in the *upc2*Δ/Δ mutants is a reduction in baseline or inducible expression of genes involved in sterol biosynthesis. In the presence of fluconazole, the *ERG11* gene product, lanosterol demethylase, is inhibited, leading to the accumulation of toxic sterol precursors and the production of alternate sterols, such as lanosterol, eburicol, obtusifoliol, 14α-methyl fecosterol, and 14α-methylergosta-8,24(28)-dien-3β,6α-diol (21). Integration of these sterols into the plasma membrane disrupts its integrity, resulting in its altered structure and function (12, 43, 44). Under these conditions, growth is inhibited but the organism remains viable (45). Fluconazole treatment also induces the expression of *ERG11* and other genes of the ergosterol biosynthesis pathway, leading to increased production not only of lanosterol demethylase but also of enzymes involved in alternate sterol production (46). However, *UPC2* disruption would reduce the level of *ERG11* expression, which would diminish the amount of ergosterol in the cell. The expression of genes encoding enzymes needed for the biosynthesis of alternate sterols would also be reduced, further compromising the organism in the face of fluconazole exposure. Indeed, the level of *ERG11* expression was reduced in the *ERG11upc2*Δ/Δ mutant as well as in 12-99*upc2*Δ/Δ as measured by real-time RT-PCR, and inducible expression of *ERG6*, *ERG24*, and *ERG27* (as well as *ERG11*) was reduced in the *upc2*Δ/Δ mutant from that in its parent strain, SC5314, upon exposure to fluconazole as measured by microarray analysis. *ERG6* and *ERG27* both encode enzymes believed to be involved in the production of alternate sterols (Fig. 7).

Of particular interest, another core set of fluconazole-induced genes found to be dependent on *UPC2* included those in the Gene Ontology process categories of iron transport (*CFL4*, *FET3*, *FET34*, *FRE10*, *FRP1*, *FTR1*) and iron homeostasis (*CFL2*, *CSA1*, *IRO1*, *RBT5*). Iron starvation has been shown to enhance the susceptibility of *C. albicans* to fluconazole (47–50). Prasad et al. observed that an *ftr1*Δ/Δ mutant, defective in high-affinity iron uptake, also exhibited enhanced susceptibility to fluconazole (47). Examination of the *ERG11* mRNA abundance and membrane ergosterol levels in the *ftr1*Δ/Δ mutant revealed reductions in both from those for wild-type cells, which led to increased membrane fluidity and consequently increased passive diffusion of fluconazole. In contrast, *ERG3*, which acts in the biosynthesis of 14α-methylergosta-8,24(28)-dien-3β,6α-diol (51), was upregulated. This phenotype reverted upon the addition of iron to the medium. A similar observation has been made for *Cryptococcus neoformans*, where disruption of the genes encoding the ferroxidase (*CFO1*) and an iron permease (*CFT1*) of the high-affinity reductive iron uptake system resulted in increased susceptibility to azole antifungals (52). In the present study, we found that when *UPC2* is intact, fluconazole exposure results in the upregulation of four genes associated with high-affinity iron uptake (*FTR1*, *FET3*, *FET34*, and *FRP1*). When *UPC2* is disrupted, fluconazole exposure results in the downregulation of these genes, suggesting that *UPC2* may be required for this process. Indeed, we found that disruption of *UPC2* in isolates SC5314 and 12-99 precluded their ability to grow under low-iron conditions. Moreover, the fluconazole susceptibility of the *upc2*Δ/Δ mutants was greatly enhanced in iron-poor medium. Further investigation of the relationship between *UPC2*, high-affinity iron uptake, and fluconazole resistance is warranted and is under way in our laboratory.

Since many enzymes in the ergosterol biosynthesis pathway require iron, it is tempting to speculate that decreased iron uptake would have a significant impact on the activities of these enzymes. This, combined with decreased expression of the genes encoding enzymes involved in ergosterol biosynthesis (*ERG11* in particular), may account for the substantial effect of *UPC2* disruption on fluconazole susceptibility observed in these strains. While further investigation is needed to determine which Upc2 targets influence susceptibility to the azole antifungals, our findings suggest that Upc2 and the transcriptional activation pathway it regulates represent potential targets for overcoming azole antifungal resistance in *C. albicans*.

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