A Gain-of-Function Mutation in the Transcription Factor Upc2p Causes Upregulation of Ergosterol Biosynthesis Genes and Increased Fluconazole Resistance in a Clinical *Candida albicans* Isolate[▽][†]

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In the pathogenic yeast *Candida albicans*, the zinc cluster transcription factor Upc2p has been shown to regulate the expression of *ERG11* and other genes involved in ergosterol biosynthesis upon exposure to azole antifungals. *ERG11* encodes lanosterol demethylase, the target enzyme of this antifungal class. Overexpression of *UPC2* reduces azole susceptibility, whereas its disruption results in hypersusceptibility to azoles and reduced accumulation of exogenous sterols. Overexpression of *ERG11* leads to the increased production of lanosterol demethylase, which contributes to azole resistance in clinical isolates of *C. albicans*, but the mechanism for this has yet to be determined. Using genome-wide gene expression profiling, we found *UPC2* and other genes involved in ergosterol biosynthesis to be coordinately upregulated with *ERG11* in a fluconazole-resistant clinical isolate compared with a matched susceptible isolate from the same patient. Sequence analysis of the *UPC2* alleles of these isolates revealed that the resistant isolate contained a single-nucleotide substitution in one *UPC2* allele that resulted in a G648D exchange in the encoded protein. Introduction of the mutated allele into a drug-susceptible strain resulted in constitutive upregulation of *ERG11* and increased resistance to fluconazole. By comparing the gene expression profiles of the fluconazole-resistant isolate and of strains carrying wild-type and mutated *UPC2* alleles, we identified target genes that are controlled by Upc2p. Here we show for the first time that a gain-of-function mutation in *UPC2* leads to the increased expression of *ERG11* and imparts resistance to fluconazole in clinical isolates of *C. albicans*.

Candida albicans is an opportunistic fungal pathogen that is responsible for a considerable portion of fungal infections in humans. In healthy people, this yeast resides as a commensal in the gastrointestinal tract, but it is capable of causing mucosal, cutaneous, and systemic infections in immunocompromised individuals (33). In patients with AIDS, oropharyngeal candidiasis, caused primarily by *C. albicans*, is the most frequent opportunistic infection (11, 20). The azole class of antifungal agents, particularly fluconazole, is effective for the management of such infections. However, with long-term treatment, azole resistance often emerges, resulting in therapeutic failure (26, 30, 35, 38, 47).

The azole antifungals bind to and inhibit the activity of lanosterol demethylase (Erg11p), a key enzyme in the ergosterol biosynthesis pathway (17). Mechanisms of resistance to the azole antifungal agents that have been described for *C. albicans* include increased expression of the gene encoding the major facilitator superfamily transporter Mdr1p and genes encoding two ATP binding cassette (ABC) transporters, Cdr1p and Cdr2p (12, 13, 26, 40, 45). Other mechanisms of resistance involve the *ERG11* gene itself. Mutations in *ERG11* that interfere with the ability of the azole to bind to its target can confer resistance (12, 16, 18, 19, 23, 35, 39, 46). Furthermore, overexpression of *ERG11* leads to the increased production of lanosterol demethylase, which can also contribute to azole resistance (12, 22, 30, 32, 35, 45).

In response to azole antifungals (i.e., fluconazole, itraconazole, and ketoconazole), wild-type *C. albicans* strains overexpress *ERG11* and other genes involved in ergosterol biosynthesis (4, 10, 24). Forced overexpression of *ERG11* or the gene encoding its regulator, *UPC2*, increases resistance to azoles, whereas mutants lacking *UPC2* show no induction of *ERG* genes in response to sterol biosynthesis inhibitors and are hypersusceptible to these drugs (1, 27, 43). They also accumulate lower levels of exogenously supplied cholesterol than those of the wild-type, demonstrating the role of *UPC2* in sterol uptake (43).

Constitutive overexpression of *CDR1* and *CDR2* in azole-resistant clinical isolates has been shown to be due to gain-of-

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† Supplemental material for this article may be found at http://ec .asm.org/.

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

Strain	Parent	Relevant characteristics or genotype[a]	Source or reference
S1		Fluconazole-susceptible isolate from patient 6	13
S2		Fluconazole-resistant isolate from patient 6	13
SC5314		Wild-type *C. albicans* model strain	14
UPC2M1A	SC5314	*upc2-1Δ::SAT1-FLIP/UPC2-2*	This study
UPC2M1B	SC5314	*UPC2-1/upc2-2Δ::SAT1-FLIP*	This study
UPC2M2A	UPC2M1A	*upc2-1Δ::FRT/UPC2-2*	This study
UPC2M2B	UPC2M1B	*UPC2-1/upc2-2Δ::FRT*	This study
UPC2M3A	UPC2M2A	*upc2-1Δ::FRT/upc2-2Δ::SAT1-FLIP*	This study
UPC2M3B	UPC2M2B	*upc2-1Δ::SAT1-FLIP/upc2-2Δ::FRT*	This study
UPC2M4A	UPC2M3A	*upc2-1Δ::FRT/upc2-2Δ::FRT*	This study
UPC2M4B	UPC2M3B	*upc2-1Δ::FRT/upc2-2Δ::FRT*	This study
UPC2M2K21A	UPC2M2A	*upc2-1Δ::FRT/UPC2^{S1-1}-caSAT1*	This study
UPC2M2K21B	UPC2M2B	*UPC2-1^{S1-1}-caSAT1/upc2-2Δ::FRT*	This study
UPC2M2K22A	UPC2M2A	*UPC2-1^{S1-1}-caSAT1/UPC2-2*	This study
UPC2M2K22B	UPC2M2B	*UPC2-1/UPC2^{S1-1}-caSAT1*	This study
UPC2M2K31A	UPC2M2A	*upc2-1Δ::FRT/UPC2^{S2-1}-caSAT1*	This study
UPC2M2K31B	UPC2M2B	*UPC2-1^{S2-1}-caSAT1/upc2-2Δ::FRT*	This study
UPC2M2K32A	UPC2M2A	*UPC2-1^{S2-1}-caSAT1/UPC2-2*	This study
UPC2M2K32B	UPC2M2B	*UPC2-1/UPC2^{S2-1}-caSAT1*	This study

[a] *SAT1-FLIP* denotes the *SAT1* flipper cassette.

function mutations in the zinc cluster transcription factor Tac1p and the loss of heterozygosity at the *TAC1* locus (6, 7). Recently, similar mutations in another zinc cluster transcription factor, Mrr1p, were found to cause constitutive overexpression of *MDR1* in fluconazole-resistant clinical isolates (31).

The comparison of gene expression in matched fluconazole-susceptible and -resistant isolates has proved to be a powerful tool to identify the resistance mechanisms of clinical *C. albicans* isolates. Such studies initially pointed to the involvement of efflux pump overexpression as well as *ERG11* overexpression in fluconazole-resistant strains (40, 45). More recently, genome-wide transcriptional profiling experiments using DNA microarrays have revealed additional alterations that might be involved in the development of drug resistance (3, 10, 24). This approach has led to the identification of the transcription factor Mrr1p, which controls the expression of the *MDR1* efflux pump (31).

In the present study, we performed genome-wide gene expression profiling of a matched pair of azole-susceptible and -resistant isolates from a series in which no overexpression of *CDR1* and *CDR2* or *MDR1* in resistant isolates was detected by Northern hybridization in a previous study (13). We observed upregulation of the *UPC2* gene, encoding a transcription factor that controls the expression of ergosterol biosynthesis genes, as well as known target genes of this transcription factor in the resistant isolate. Here we show for the first time that a gain-of-function mutation in *UPC2* leads to the increased expression of *ERG11* and imparts resistance to fluconazole in *C. albicans*.

MATERIALS AND METHODS

Strains and growth conditions. *C. albicans* strains used in this study are listed in Table 1. All strains were stored as frozen stocks with 15% glycerol at −80°C and subcultured on yeast-peptone-dextrose (YPD) agar plates (10 g yeast extract, 20 g peptone, 20 g dextrose, 15 g agar per liter) at 30°C. For routine growth of the strains, YPD liquid medium was used. The selection of nourseothricin-resistant transformants and the isolation of nourseothricin-sensitive derivatives in which the *SAT1* flipper was excised by FLP-mediated recombination was performed as described previously (36).

Plasmid constructions. The coding region and flanking sequences of the *UPC2* alleles from isolates S1 and S2 were amplified by PCR with the primers UPC2-3A and UPC2-4A, which bind in the *UPC2* upstream and downstream regions, respectively (for primer sequences, see Table 2). The PCR products were digested at the introduced SacI and ApaI sites and cloned in the vector pBluescript to generate plasmids pUPC2S1-1, pUPC2S1-2, pUPC2S2-1, and pUPC2S2-2. Several clones from independent PCRs were sequenced to ensure that both *UPC2* alleles were obtained from each isolate and to exclude PCR errors.

A *UPC2* deletion cassette was generated in the following way: a SacI-SacII fragment containing *UPC2* upstream sequences from positions −373 to +15 with respect to the start codon was amplified from the genomic DNA of strain SC5314 with the primers UPC2-3A and UPC2-5, and an XhoI-SacI fragment containing *UPC2* downstream sequences from positions +2097 to +2437 was amplified with the primers UPC2-6 and UPC2-4A. The *UPC2* upstream and downstream fragments were cloned on both sides of the *SAT1* flipper cassette in plasmid pSFS2 (36) to result in pUPC2M2, in which the *UPC2* coding region from positions +16 to +2096 (44 bp before the stop codon) is replaced by the *SAT1* flipper (see Fig. 1A).

To express the mutated *UPC2-1* allele from isolate S2 (*UPC2^{S2-1}*) and the corresponding nonmutated *UPC2-1* allele from isolate S1 (*UPC2^{S1-1}*) in the SC5314 background, a *UPC2* downstream fragment was first amplified with the primers UPC2-7 and UPC2-4A, digested at the introduced PstI and ApaI sites, and cloned behind the *Candida albicans*-adapted *SAT1* (ca*SAT1*) selection marker in pZCF36K1 (31) to obtain pUPC2K1. The SacI-NdeI fragments from pUPC2S1-1 and pUPC2S2-1 (positions −373 to +1949 in *UPC2*) were then cloned together with an NdeI-BglII fragment from pTET6-UPC2 (J. Morschhäuser, unpublished results) containing the C-terminal part of the *UPC2* open reading frame (ORF) (positions +1950 to +2139) in SacI-/BglII-digested pUPC2K1 to result in pUPC2K2 and pUPC2K3, respectively (see Fig. 1B).

The coding region of the *ERG11* gene was amplified by PCR from genomic DNA using primers ERG11seqF (5′-ATGGCTATTGTTGAAACTGTCATTG-3′) and ERG11seqR (5′-TCTGAACACTGAATCGAAAG-3′). Several independent PCRs from each isolate (isolates S1 and S2) were performed, and these products were cloned into plasmid pCR2.1 (Invitrogen, Carlsbad, CA). The

TABLE 2. Primers used for *UPC2* cassette construction

Primer	Sequence[a]
UPC2-3A	5′-AACA<u>GAGCTC</u>TACGTTATTCAGCTTTCC-3′
UPC2-4A	5′-TTAT<u>GGGCCC</u>CACAGTAACGAATCACATTTGTG-3′
UPC2-5	5′-ATTC<u>CCGCGG</u>CACTGTCATCATCATAAATGGC-3′
UPC2-6	5′-AGTA<u>CTCGAG</u>ACTTAATGCAAGGTGATAATGG-3′
UPC2-7	5′-AGTA<u>CTGCAG</u>ACTTAATGCAAGGTGATAATGG-3′

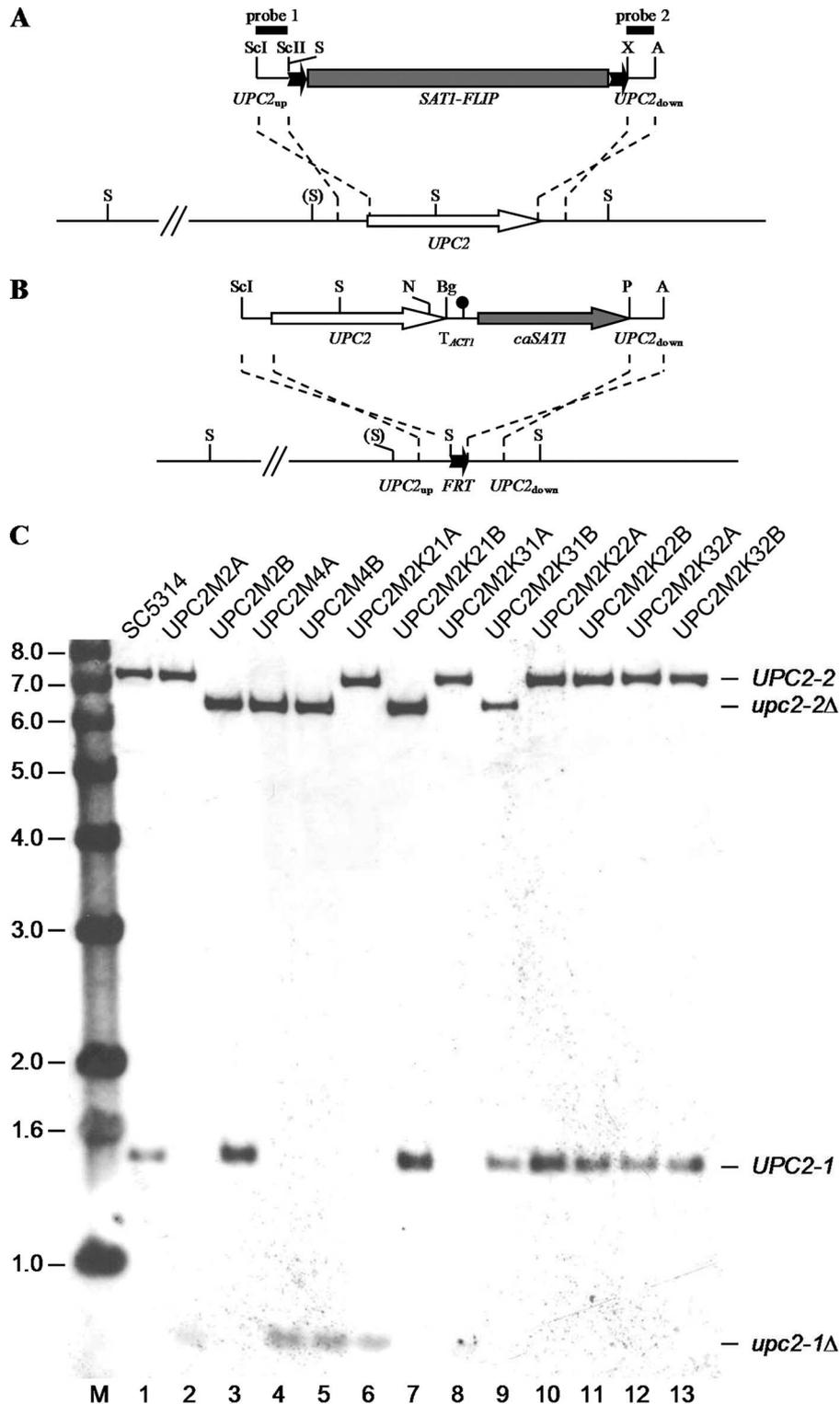[a] Restriction sites introduced into primers are underlined.

FIG. 1. Construction of $upc2\Delta$ mutants and strains expressing the $UPC2^{S1-1}$ and $UPC2^{S2-1}$ alleles. (A) Structure of the deletion cassette from plasmid pUPC2M2 (top), which was used to delete the $UPC2$ ORF in strain SC5314, and genomic structure of the $UPC2$ locus in the parental strain (bottom). The $UPC2$ coding region is represented by the white arrow and the upstream ($UPC2_{up}$) and downstream ($UPC2_{down}$) regions by the solid lines. The $SAT1$ flipper cassette ($SAT1$-$FLIP$) is represented by the gray rectangle bordered by FRT sites (black arrows). The 34-bp FRT sites are not drawn to scale. The probes used for Southern hybridization analysis of the mutants are indicated by the black bars. (B) Structure of the DNA fragments from plasmids pUPC2K2 and pUPC2K3 (top), which were used for integration of the $UPC2^{S1-1}$ and $UPC2^{S2-1}$ alleles, respectively, into the remaining wild-type $UPC2$ locus (not shown) or the disrupted $upc2\Delta$ locus of the heterozygous $upc2$ mutants (bottom) using the ca$SAT1$ selection marker (gray arrow). T_{ACT1}, transcription termination sequence of the $ACT1$ gene. A, ApaI; Bg, BglII; N, NdeI; P, PstI; S, SpeI; ScI, SacI; ScII, SacII; X, XhoI. Only relevant restriction sites are given for panels A and B. The SpeI site shown in parentheses is present only on the

double-stranded DNA sequence from the full ORF was obtained for each clone from each isolate.

C. albicans transformation. C. albicans strains were transformed by electroporation (21) with the gel-purified SacI-ApaI fragments from pUPC2M2 (for UPC2 deletion) and pUPC2K2 or pUPC2K3 (for introduction of the UPC2[S1-1] and UPC2[S2-1] alleles, respectively). Nourseothricin-resistant transformants were selected on YPD agar plates containing 200 μg ml^{-1} nourseothricin (Werner Bioagents, Jena, Germany) as described previously (36). The correct genomic integration of all constructs was confirmed by Southern hybridization.

Isolation of genomic DNA and Southern hybridization. Genomic DNA from C. albicans was isolated as described previously (29). Ten micrograms of DNA was digested with SpeI, separated on a 1% agarose gel, and after ethidium bromide staining, transferred by vacuum blotting onto 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 (GE Healthcare, Braunschweig, Germany) according to the instructions of the manufacturer.

Drug susceptibility tests. Overnight cultures of the strains grown in YPD medium were serially 10-fold diluted, and 5 μl of the 10^{-1} to 10^{-5} dilutions was spotted on YPD agar plates with or without 1, 2, 5, and 10 μg ml^{-1} fluconazole or terbinafine and incubated for 2 days at 30°C. The MICs of fluconazole were also determined by broth microdilution as described by the Clinical and Laboratory Standards Institute (37).

Construction of C. albicans Affymetrix expression array. We developed a new Affymetrix custom expression array (CAN07, 49-5241 array format with 11-μm features) for C. albicans. The nucleotide sequences corresponding to 6,165 ORFs for C. albicans were downloaded from the Galar Fungail European Consortium (assembly 6; http://www.pasteur.fr/Galar_Fungail/CandidaDB). We planned to design two separate probe sets for each ORF, each consisting of 11 perfect matches and 11 mismatches overlapping 25-bp oligonucleotides, to the 3' 600-bp region. For ORFs less than 600 bp in length, the sequence was divided into two equal segments for subsequent design procedures. For quality control and normalization purposes, we made additional control probe sets corresponding to GAPDH (glyceraldehyde-3-phosphate dehydrogenase) (1 probe set), actin (1 probe set), and the 5' and middle region of the Mdr1p (Bmr1p) transcript in addition to the standard Affymetrix controls (BioB, -C, and -D, cre, DAP, PHE, LYS, and THR). The probe selection was performed by the Chip Design Group at Affymetrix, Inc., using their proprietary algorithm to calculate probe set scores, which includes a probe quality metric, a cross-hybridization penalty, and a gap penalty. The probe sets were then examined for cross-hybridization against all other sequences in the C. albicans genome as well as a number of constitutively expressed genes and rRNA from other common organisms. Consequently, for some target regions, we were not able to design high-quality probe sets. In the end, the GeneChip contained 10,014 probe sets, corresponding to 5,806 unique ORFs. Two probes were represented for 4,208 ORFs, allowing two independent measurements of the mRNA level for that particular gene.

RNA preparation for microarrays. Total RNA was isolated using the hot sodium dodecyl sulfate-phenol method (41). Frozen cells were suspended in 12 ml of 50 mM sodium acetate (pH 5.2), 10 mM EDTA at room temperature, after which 1 ml of 20% sodium dodecyl sulfate and 12 ml of acid phenol (Fisher Scientific) were added. This mixture was incubated for 10 min at 65°C with mixing each minute, cooled on ice for 5 min, and centrifuged for 15 min at $12,000 \times g$. Supernatants were transferred to new tubes containing 15 ml of chloroform, mixed, and centrifuged at $200 \times g$ for 10 min. The aqueous layer was removed to new tubes, and RNA was precipitated with 1 volume of isopropanol and 0.1 volume of 2 M sodium acetate (pH 5.0) and then collected by centrifugation at $17,000 \times g$ for 35 min at 4°C. The RNA pellet was suspended in 10 ml of 70% ethanol, collected again by centrifugation, and suspended in nuclease-free water.

cRNA synthesis and labeling. Immediately prior to cDNA synthesis, the purity and concentration of RNA samples were determined by A_{260}/A_{280} readings, and RNA integrity was determined by capillary electrophoresis using the RNA 6000 Nano Laboratory-on-a-Chip kit and Bioanalyzer 2100 (Agilent Technologies) as per the manufacturer's instructions. First- and second-strand cDNA was synthesized from 15 μg total RNA using the SuperScript double-stranded cDNA synthesis kit (Invitrogen) and the oligo(dT24-T7) primer (PrOligo) according to

the manufacturer's instructions. cRNA was synthesized and labeled with biotinylated UTP and CTP by in vitro transcription using the T7 promoter-coupled double-stranded cDNA as a template and the Bioarray HighYield RNA transcript labeling kit (ENZO Diagnostics). Double-stranded cDNA synthesized from the previous steps was washed twice with 70% ethanol and suspended in 22 μl of RNase-free water. The cDNA was incubated as recommended by the manufacturer with reaction buffer, biotin-labeled ribonucleotides, dithiothreitol, RNase inhibitor mix, and T7 RNA polymerase for 5 h at 37°C. The labeled cRNA was separated from unincorporated ribonucleotides by passing through a Chroma Spin-100 column (Clontech) and ethanol precipitated at −20°C overnight.

Oligonucleotide array hybridization and analysis. The cRNA pellet was suspended in 10 μl of RNase-free water, and 10 μg was fragmented by ion-mediated hydrolysis at 95°C for 35 min in 200 mM Tris-acetate (pH 8.1), 500 mM potassium acetate, 150 mM magnesium acetate. The fragmented cRNA was hybridized for 16 h at 45°C to the C. albicans NimbleExpress GeneChip arrays. Arrays were washed at 25°C with 6× SSPE (1× SSPE is 0.18 M NaCl, 10 mM NaH$_2$PO$_4$, and 1 mM EDTA [pH 7.7]), 0.01% Tween 20, followed by a stringent wash at 50°C with 100 mM MES [2-(N-morpholino)ethanesulfonic acid], 0.1 M NaCl, 0.01% Tween 20. Hybridizations and washes employed the Affymetrix Fluidics station 450 using their standard EukGE-WS2v5 protocol. The arrays were then stained with phycoerythrin-conjugated streptavidin (Molecular Probes), and the fluorescence intensities were determined using the GCS 3000 high-resolution confocal laser scanner (Affymetrix). The scanned images were analyzed using software resident in GeneChip operating system v2.0 (Affymetrix). Sample loading and variations in staining were standardized by scaling the average of the fluorescent intensities of all genes on an array to a constant target intensity (250). The signal intensity for each gene was calculated as the average intensity difference, represented by [Σ(PM − MM)/(number of probe pairs)], where PM and MM denote perfect-match and mismatch probes.

Microarray data analysis. The scaled gene expression values from GeneChip operating system v2.0 software were generated using the MAS5.0 algorithm. The value of each probe set is generated using Tukey's biweight computation. The algorithm considers the contribution of each match and mismatch probe corrected for the background of the average of the probe set. A weighted mean is then calculated for each probe pair. The raw signal values are then log$_2$ transformed, and the array is normalized to this value. Probe sets were deleted from subsequent analysis if they were determined to be absent by the Affymetrix criterion. Pairwise comparison of gene expression level was performed for each matched experiment. Among direct comparisons between matched clinical isolates or between strains, genes were considered to be differentially expressed if their change in expression was 1.5-fold in both independent experiments. Data files for each scanned chip were submitted to the Gene Expression Omnibus database (www.ncbi.nlm.nih.gov/geo/). The accession number for the series is GSE11320.

Quantitative real-time PCR. An aliquot of the RNA preparations from the samples used in the microarray experiments was saved for quantitative real-time reverse transcription-PCR (RT-PCR) follow-up studies. First-strand cDNAs were synthesized from 2 μg of total RNA in a 21-μl reaction volume using the SuperScript first-strand synthesis system for RT-PCR (Invitrogen) in accordance with the manufacturer's instructions. Quantitative real-time PCRs were performed in triplicate using the 7000 sequence detection system (Applied Biosystems). Independent PCRs were performed using the same cDNA for both the gene of interest and the 18S rRNA, using the SYBR green PCR master mix (Applied Biosystems). Gene-specific primers were designed for the gene of interest and the 18S rRNA using Primer Express software (Applied Biosystems) and the Oligo analysis and plotting tool (Qiagen) and are shown in Table 3. The PCR conditions consisted of AmpliTaq Gold activation at 95°C for 10 min, followed by 40 cycles of denaturation at 95°C for 15 s and annealing/extension at 60°C for 1 min. A dissociation curve was generated at the end of each PCR cycle to verify that a single product was amplified by using software provided with the 7000 sequence detection system. The change in fluorescence of SYBR green I dye in every cycle was monitored by the system software, and the threshold cycle (C_T) above the background for each reaction was calculated. The C_T value of 18S rRNA was subtracted from that of the gene of interest to obtain a ΔC_T value.

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chromosome containing the UPC2-1 allele in strain SC5314. (C) Southern hybridization of SpeI-digested genomic DNA of the wild-type strain SC5314 (lane 1), heterozygous (lanes 2 and 3) and homozygous (lanes 4 and 5) upc2Δ mutants, and strains with reinserted UPC2 alleles (lanes 6 to 13) with the UPC2-specific probe 1. The sizes (in kb) of the 1-kb ladder (lane M), which was also labeled, are shown on the left side of the blot. The positions of the original wild-type UPC2 alleles and the inactivated upc2Δ alleles are shown on the right side of the blot.

TABLE 3. Primers used for quantitative real-time PCR analysis

Gene name	Direction[a]	Primers	Amplicon size
18S	F	5′-CACGACGGAGTTTCACAAGA-3′	135
	R	5′-CGATGGAAGTTTGAGGCAAT-3′	
CDR1	F	5′-ATTCTAAGATGTCGTCGCAAGATG-3′	140
	R	5′-AGTTCTGGCTAAATTCTGAATGTTTTC-3′	
CDR2	F	5′-TAGTCCATTCAACGGCAACATT-3′	76
	R	5′-CACCCAGTATTTGGCATTGAAA-3′	
MDR1	F	5′-ACATAAATACTTTGCCCATCCAGAA-3′	82
	R	5′-AAGAGTTGGTTTGTAATCGGCTAAA-3′	
ERG11	F	5′-TTTAGTTTCTCCAGGTTATGCTCAT-3′	100
	R	5′-ATTAGCTTTGGCAGCAGCAGTA-3′	
UPC2	F	5′-TCCATCCTTGACCCCTAGTCCT-3′	52
	R	5′-CGGCTGAGTTTTGATGTCTTGA-3′	

[a] F, forward; R, reverse.

The ΔC_T value of an arbitrary calibrator (e.g., an untreated sample) was subtracted from the ΔC_T value of each sample to obtain a $\Delta\Delta C_T$ value. The gene expression level relative to the calibrator was expressed as $2^{-\Delta\Delta CT}$. Experiments were performed in triplicate. Statistical analysis was performed using Student's t test. The statistical significance threshold was fixed at an α value of 0.05.

Bioinformatics analysis. The promoter sequences (1 kb upstream of the start codon) were downloaded from the Candida Genome Database (CGD) (http://www.candidagenome.org). Using the fuzznuc application from EMBOSS (http://emboss.bioinformatics.nl), the promoter sequences were examined for putative sterol regulatory element (SRE) motifs.

Nucleotide sequence accession numbers. The coding sequences of the *UPC2* alleles described in this study have been deposited in GenBank with the following accession numbers: EU583451 (*UPC2*[S1-1]), EU583452 (*UPC2*[S1-2]), and EU583453 (*UPC2*[S2-1]).

RESULTS

Expression of the transcription factor *UPC2* is coordinately upregulated with *ERG11* in azole-resistant *C. albicans* clinical isolate S2. The *C. albicans* isolates S1 (fluconazole susceptible) and S2 (fluconazole resistant) were recovered from an AIDS patient who failed fluconazole therapy for treatment of oropharyngeal candidiasis; they are the first two isolates of a series in which no overexpression of the transporter genes *CDR1*, *CDR2*, and *MDR1* was detected by Northern hybridization (13). In order to identify genes that are differentially expressed between these two isolates, we compared their transcriptional profiles using DNA microarrays. As can be seen in Table S1 and Table S2 of the supplemental material, 138 genes were upregulated and 173 genes were downregulated in isolate S2 compared to S1. Interestingly, among the upregulated genes were several that are involved in ergosterol biosynthesis (*ERG1*, *ERG3*, *ERG4*, *ERG7*, *ERG24*, *ERG26*, *ERG27*, *POT14*, and *NCP1*) as well as *UPC2*, which encodes a transcription factor that regulates ergosterol biosynthesis genes and induces their expression in the presence of azoles (27, 34, 43). Furthermore, the major facilitator superfamily transporter gene *MDR1* was also upregulated in S2. *ERG11*, the gene encoding the target of fluconazole, was not found to be differentially expressed between these isolates by microarray analysis. However, real-time RT-PCR analysis revealed increased expression of *ERG11* in isolate S2 compared to that in S1. Moreover, real-time RT-PCR confirmed the upregulation of *UPC2* and *MDR1* and no change in the expression of *CDR1*. While we observed downregulation of *CDR2* by using real-time RT-PCR, this was not observed by using microarray analysis (Fig. 2).

Azole-resistant *C. albicans* clinical isolate S2 contains a mutation in *UPC2*. The finding that the expression of ergosterol
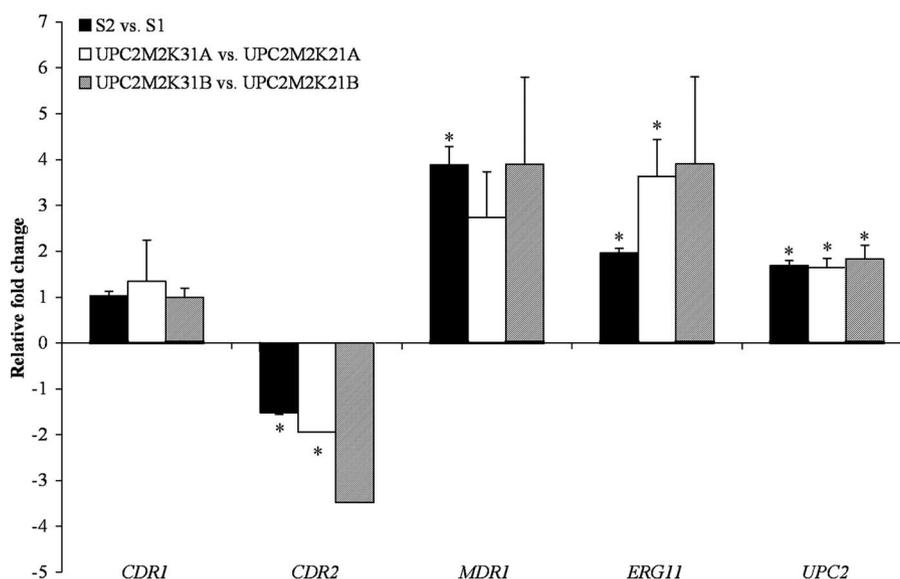

FIG. 2. Quantitative real-time RT-PCR for genes of interest. Shown are the relative *n*-fold changes in gene expression in the clinical isolates (S2 versus S1) and in two pairs of transformants expressing the *UPC2*[S2-1] or *UPC2*[S1-1] alleles in the SC5314 background (UPC2M2K31A versus UPC2M2K21A and UPC2M2K31B versus UPC2M2K21B). Error bars represent the standard error of the mean. Asterisks denote statistical significance by Student's t test ($P \leq 0.05$). vs., versus.

biosynthesis genes and their regulator *UPC2* was increased in the fluconazole-resistant isolate S2 suggested that this isolate might contain a mutation in *UPC2* that causes constitutive upregulation of its target genes as well as of *UPC2* itself. We therefore cloned and sequenced the *UPC2* alleles of isolates S1 and S2. Two polymorphic *UPC2* alleles were found in the susceptible isolate S1. The coding sequence of allele 1 corresponds to that of *UPC2* of the *C. albicans* reference strain SC5314 used for genome sequencing. It contains four silent nucleotide substitutions (T1338C, C1392T, C1410A, and C1539T) that are not present in ORFs 19.391 and 19.8021, which encode *UPC2* (http://www.candidagenome.org), but the same polymorphisms have been described in an earlier version of the genome sequence of this strain (15). Allele 2 differed from allele 1 by 19 nucleotide substitutions, 4 of which resulted in amino acid exchanges (R68K, I142S, S190N, and S228N). Isolate S2 contained the same two alleles; however, while allele 2 was identical to allele 2 of isolate S1, allele 1 contained a G1943A mutation that was not present in allele 1 of isolate S1 and which resulted in a G648D substitution in the encoded protein. Direct sequencing of the PCR products confirmed the absence of the G1943A mutation in the *UPC2* alleles of isolate S1 and heterozygosity for this mutation in isolate S2.

Azole-resistant *C. albicans* clinical isolate S2 contains mutations in *ERG11*. In order to determine whether there were any mutations in *ERG11* which could contribute to fluconazole resistance, we cloned and sequenced the *ERG11* gene from isolates S1 and S2. From the alleles recovered, we were able to determine that there were two nucleotide changes in isolate S2 that were not present in isolate S1: a G-A transition at nucleotide 919 (relative to the start codon ATG) resulting in a G307S amino acid change and an additional G448E change due to a G-A transition at nucleotide 1341. Both of these mutations have been found previously in other fluconazole-resistant isolates, and the G307S mutation was shown to cause increased fluconazole resistance upon expression in *Saccharomyces cerevisiae* (25, 35).

The G648D mutation in Upc2p confers increased resistance to ergosterol biosynthesis inhibitors. To test whether the G648D substitution in Upc2p is a gain-of-function mutation that is responsible for the increased fluconazole resistance of isolate S2, we expressed the mutated *UPC2-1* allele from isolate S2 (*UPC2*[S2-1]) in the fluconazole-susceptible *C. albicans* strain SC5314, either alone or in the presence of a wild-type *UPC2* allele. For this purpose, we first deleted one of the *UPC2* alleles of strain SC5314 using the *SAT1*-flipping strategy (36). The *UPC2* alleles of strain SC5314 can be distinguished by an SpeI restriction site polymorphism, and we generated two independent heterozygous mutants (strains UPC2M2A and -B) in which one or the other *UPC2* allele was deleted (Fig. 1A and C, lanes 1 to 3). A second round of gene deletion resulted in the homozygous *upc2*Δ mutants UPC2M4A and -B (Fig. 1C, lanes 4 and 5), which were included for comparison in the following experiments. The mutated *UPC2*[S2-1] allele was then either substituted for the remaining wild-type *UPC2* allele in the heterozygous mutants to generate strains UPC2M2K31A and -B (Fig. 1C, lanes 8 and 9) or inserted into the already disrupted *upc2*Δ locus (see Fig. 1B) to generate strains UPC2M2K32A and -B (Fig. 1C, lanes 12 and 13). Replacement of the wild-type allele by the mutated *UPC2*[S2-1] allele in

strains UPC2M2K31A and -B was confirmed by reamplification and sequencing. As a control, the otherwise identical but nonmutated *UPC2-1* allele from isolate S1 (*UPC2*[S1-1]) was integrated in the same way, generating strains UPC2M2K21A and -B and UPC2M2K22A and -B (Fig. 1C, lanes 6, 7, 10, and 11).

We then compared the susceptibilities of the strains to two different ergosterol biosynthesis inhibitors, fluconazole, which inhibits lanosterol demethylase, and terbinafine, which inhibits squalene epoxidase. In line with previous reports (27, 43), the homozygous *upc2*Δ mutants were highly susceptible to fluconazole and terbinafine, and the heterozygous mutants also showed enhanced susceptibility to both inhibitors compared with the wild-type parental strain SC5314 (Fig. 3, top panels). The strains expressing the mutated *UPC2*[S2-1] allele exhibited increased resistance to fluconazole compared with the wild-type parental strain, irrespective of the presence of an additional wild-type allele (Fig. 3, middle panels). In contrast, replacement of the remaining wild-type allele by the nonmutated *UPC2*[S1-1] allele had no effect on the susceptibility of the heterozygous mutants, and introduction of this allele into the disrupted *upc2*Δ locus restored fluconazole resistance to wild-type levels (Fig. 3, bottom panels). Transformants expressing the mutated *UPC2*[S2-1] allele also exhibited slightly increased resistance to terbinafine compared with transformants containing the nonmutated *UPC2*[S1-1] allele (Fig. 3, right panels). Similar results were observed for all concentrations tested. These results demonstrate that the G648D mutation in Upc2p confers increased resistance to ergosterol biosynthesis inhibitors.

Additional susceptibility testing was undertaken using CLSI methodology (Table 4). The MIC of fluconazole for isolate S2 was 64 μg/ml, whereas that for isolate S1 was 1 μg/ml. The fluconazole MIC for strain SC5314 was 0.25 μg/ml. Similar to results observed by spot assay, the homozygous *upc2*Δ mutants were more susceptible to fluconazole then was the wild-type parental strain SC5314. However, the heterozygous mutants exhibited the same MIC as strain SC5314. Strains expressing a single copy of the UPC2[S1-1] allele exhibited MICs identical to those of the parental wild-type strain SC5314, whereas the strains expressing the mutated UPC2[S2-1] allele exhibited a fourfold increase in resistance to fluconazole compared with the wild-type parental strain. While this change is significant, it is not in and of itself sufficient to account for the high-level fluconazole resistance observed in isolate S2. As noted above, the level of fluconazole resistance observed in isolate S2 can be attributed in part to mutations in *ERG11* that result in G307S and G448E amino acid substitutions.

Identification of *UPC2* target genes. In order to identify target genes of Upc2p whose expression is affected by the G648D gain-of-function mutation, we compared the gene expression profiles of strains expressing either a wild-type *UPC2* allele (*UPC2*[S1-1]) or its hyperactive, mutated counterpart (*UPC2*[S2-1]) in the SC5314 background. As shown in Table S1 in the supplemental material, 93 genes were consistently upregulated in the transformants expressing the *UPC2*[S2-1] allele. Thirty-two of these were also upregulated in the azole-resistant isolate S2 compared to isolate S1 (Table 5). Sixty-six genes were consistently downregulated in the transformants expressing the *UPC2*[S2-1] allele (see Table S2 in the supplemental material). Of these, only 11 were also downregulated in isolate
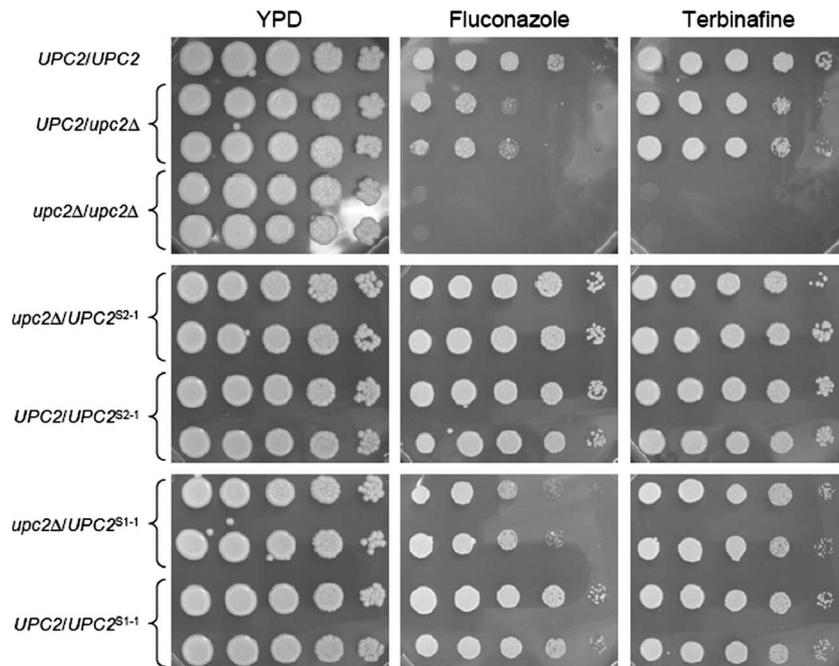
FIG. 3. Susceptibilities to fluconazole and terbinafine of the wild-type strain SC5314 (*UPC2/UPC2*), heterozygous (*UPC2/upc2Δ*) and homozygous (*upc2Δ/upc2Δ*) *upc2Δ* mutants, and strains expressing the mutated *UPC2*[S2-1] allele or the nonmutated *UPC2*[S1-1] allele in the absence (*upc2Δ/UPC2*[S2-1] and *upc2Δ/UPC2*[S1-1]) or presence (*UPC2/UPC2*[S2-1] and *UPC2/UPC2*[S1-1]) of a wild-type *UPC2* allele. Serial 10-fold dilutions of YPD overnight cultures of the strains were spotted on YPD plates without or with 2 μg ml⁻¹ fluconazole or 2 μg ml⁻¹ terbinafine and incubated for 2 days at 30°C.

S2 (Table 6). We considered those genes that were differentially expressed in both the clinical isolate S2 and transformants expressing the *UPC2*[S2-1] allele to represent a core set of Upc2p target genes. Among these, 18 genes contained at least one SRE motif, the putative binding site of Upc2p (20, 34), within their promoters (Table 7). As was observed for isolate S2 compared to S1, real-time RT-PCR analysis revealed the increased expression of *ERG11* in strains UPC2M2K31A and UPC2M2K31B compared to UPC2M2K21A and UPC2M2K21B, respectively. Moreover, real-time RT-PCR confirmed the upregulation of *UPC2* and *MDR1* and no change in the expression of *CDR1*. Downregulation of *CDR2* was observed by real-time RT-PCR, but this was not observed by microarray analysis (Fig. 2).

Disruption of *UPC2* in wild-type strain SC5314 also provided us with the opportunity to identify genes whose baseline expression requires Upc2p under the growth conditions used in our study. We therefore compared the gene expression profiles of strain SC5314 and the two independently constructed homozygous, *upc2Δ* mutants, UPC2M4A and UPC2M4B. Surprisingly, only five genes were expressed at higher levels in strain SC5314 then in the disruption mutants (see Table S3 in the supplemental material), suggesting that Upc2p is not required for baseline expression of the core target genes listed in Table 5 and Table 6. Previous studies have shown that the disruption of *UPC2* in *C. albicans* did not affect the basal expression of *ERG2*, *ERG7*, *ERG11*, and *ERG25* (27, 43). Interestingly, the disruption of *UPC2* in strain SC5314 resulted in the upregulation of 41 genes (see Table S4 in the supplemental material). This raises the possibility that Upc2p may also have a role as a transcriptional repressor. Alternatively, this may simply reflect compensatory changes in gene expression.

DISCUSSION

C. albicans Upc2p is the sole homolog of two zinc cluster transcription factors in *S. cerevisiae*, Upc2p and Ecm22p, which regulate the expression of genes involved in sterol biosynthesis and the uptake of sterols from the environment (8, 27, 42–44). In *S. cerevisiae*, a G888D mutation in Upc2p causes the uptake of exogenously supplied sterols under aerobic conditions, under which sterol uptake is normally repressed, and increased transcriptional activity (8, 9). An analogous mutation, G790D, in Ecm22p resulted in a similar phenotype (9, 42). Sequence alignment showed that the G648D mutation that we identified in one of the *UPC2* alleles of *C. albicans* isolate S2 corresponds

TABLE 4. Fluconazole MICs of studied strains

Strain	Fluconazole MIC (μg/ml)
S1	1
S2	>64
SC5314	0.25
UPC2M2A	0.25
UPC2M2B	0.25
UPC2M4A	0.125
UPC2M4B	0.125
UPC2M2K21A	0.25
UPC2M2K21B	0.25
UPC2M2K31A	1
UPC2M2K31B	1

TABLE 5. Genes upregulated by the G648D mutation in Upc2p

CandidaDB name[a]	Common name[b]	orf19 no.[c]	GO annotation (molecular function)[d]	S2 vs S1		Strain A		Strain B	
				Expt 1	Expt 2	Expt 1	Expt 2	Expt 1	Expt 2
BMR1	*MDR1*	orf19.5604	Multidrug transporter activity	2.1	3.7	5.4	3.5	2.5	5.1
DDR48	*DDR48*	orf19.4082		7.1	8.0	2.4	3.4	2.4	3.8
ECM3	*ECM3*	orf19.1563		1.6	1.5	1.7	4.1	2.9	3.2
ERG1	*ERG1*	orf19.406	Squalene monooxygenase activity	2.4	1.6	1.5	1.7	1.6	1.8
ERG24	*ERG24*	orf19.1598	Delta 14-sterol reductase activity	2.3	2.0	3.2	2.9	3.5	2.9
ERG26	*ERG26*	orf19.2909	C-3 sterol dehydrogenase (C-4 sterol decarboxylase) activity	2.0	1.6	2.0	2.0	1.9	2.0
ERG27	*ERG27*	orf19.3240	3-Keto sterol reductase activity	2.3	2.1	2.2	3.6	3.0	2.8
FEN2		orf19.5535	Pantothenate transporter activity	2.2	2.2	2.3	2.7	2.7	2.8
FTH1	*FTH1*	orf19.4802		1.5	1.5	1.8	4.3	2.4	3.7
IDP2	*IDP2*	orf19.3733	Isocitrate dehydrogenase (NADP$^+$) activity	3.6	2.1	1.8	1.6	1.8	1.7
IFA24.3	*FGR51*	orf19.156		1.8	1.9	1.7	1.7	1.6	1.7
NCP1	*NCP1*	orf19.2672	NADPH-hemoprotein reductase activity	2.5	1.5	1.9	2.2	1.7	1.8
QRI8		orf19.7329	Ubiquitin-protein ligase activity	1.5	2.0	1.6	1.7	1.5	1.7
TEF41	*TEF4*	orf19.2652	Translation elongation factor activity	4.1	3.4	18.8	28.9	19.0	14.9
IPF12101	*PGA7*	orf19.5635		1.6	2.7	5.4	8.8	6.0	9.0
IPF1218	*SOD4*	orf19.2062	Copper-zinc superoxide dismutase activity	2.3	2.3	2.2	1.7	1.8	1.6
IPF14119	*DAG7*	orf19.4688		1.8	1.6	1.7	1.6	1.7	1.8
IPF14652		orf19.2653		4.3	1.6	1.7	2.8	4.3	1.9
IPF18080		orf19.7357		1.9	1.6	1.5	1.8	1.6	1.6
IPF20010		orf19.5799		2.0	2.2	2.2	2.8	1.6	2.0
IPF20169	*PGA10*	orf19.5674	Heme binding	4.1	10.8	5.6	13.0	7.0	11.1
IPF2903		orf19.7456		1.8	2.3	2.0	1.7	2.2	1.6
IPF3352		orf19.4013		2.3	2.3	2.0	2.5	1.9	2.0
IPF4059		orf19.1865		1.6	2.1	2.7	2.5	1.9	2.4
IPF4721		orf19.3737		1.5	1.6	2.3	1.5	1.8	1.6
IPF6298		orf19.1964		3.0	1.6	1.7	1.7	2.3	1.7
IPF6518		orf19.1691		3.3	5.3	1.7	2.3	2.0	2.7
IPF7289	*UPC2*	orf19.391	Transcription factor activity	1.6	2.4	2.1	2.5	2.4	2.5
IPF7397		orf19.1800		2.9	2.6	1.8	1.7	1.6	2.2
IPF7432		orf19.6219		2.0	2.3	1.9	2.4	2.1	2.2
IPF867	*SET3*	orf19.7221	NAD-dependent histone deacetylase activity	2.1	1.7	3.6	2.9	3.2	2.4
IPF8970		orf19.496		1.9	2.5	2.2	1.9	1.6	1.8

Note: columns under "Fold increase in expression" — S2 vs S1, and UPC2^{S2-1} vs UPC2^{S1-1} (Strain A and Strain B).

[a] Gene name from CandidaDB (http://genolist.pasteur.fr/CandidaDB/).
[b] Gene name from CGD (http://www.candidagenome.org/).
[c] orf19 nomenclature according to assembly 19.
[d] Gene Ontology (GO) (4) annotation found at CGD (http://www.candidagenome.org/).

exactly to the G888D and G790D mutations that result in hyperactive *UPC2* and *ECM22* alleles, respectively, in *S. cerevisiae*. It is striking that the same mutation has been selected for in a clinical *C. albicans* isolate during fluconazole therapy.

S. cerevisiae Upc2p and Ecm22p have been shown to regulate *ERG2* and *ERG3*. An 11-bp SRE was identified in the promoters of *ERG2* and *ERG3*, and Upc2p and Ecm22p directly bound to this element. The SRE was also found in the promoter regions of other ergosterol biosynthesis genes (44). Many *C. albicans ERG* genes contain the known *S. cerevisiae* SRE motif in their promoters, and the DNA binding domain of *C. albicans* Upc2p was shown to bind to SREs contained in the *ERG2* promoter of *S. cerevisiae* and *C. albicans* (27, 43). Of the 12 *ERG* genes that were found to be upregulated in isolate S2 or by the *UCP2*$^{S2-1}$ allele in SC5314 in our present study, 9 (*ERG1*, *ERG2*, *ERG5*, *ERG6*, *ERG10*, *ERG11*, *ERG24*, *ERG26*, and *ERG27*) have been previously reported to contain the SRE core motif, TCGTATA (27, 43). In addition, *ERG7* and *UPC2* were identified as having a putative SRE (27). While the *ERG7* promoter does not contain the SRE core motif, we

did find the TCGTATA motif in *UPC2* itself, suggesting that this gene may be autoregulated.

In addition to the *ERG* genes, a host of other genes are regulated by *UPC2*. One gene of particular interest that was found to be upregulated by the G648D mutation in Upc2p is *MDR1*. Constitutive overexpression of this efflux pump leads to fluconazole resistance and represents a major mechanism of resistance in clinical isolates (2, 12, 13, 26, 35, 40, 45). Previous Northern hybridization analyses showed no *MDR1* upregulation in isolates of the S series, although isolate S2 itself was not tested in that study (13). However, in the present study, microarray analysis revealed *MDR1* to be upregulated in isolate S2 by between two- and fourfold, a result that was confirmed by real-time RT-PCR. This level of upregulation is modest in comparison to that seen in isolates in which *MDR1* has been shown to contribute to fluconazole resistance. Such isolates exhibit increased expression of *MDR1* on the order of 100-fold (31). It is therefore unlikely that enhanced expression of *MDR1* contributes significantly to fluconazole resistance in isolate S2. Moreover, as *MDR1*-mediated resistance appears to be

TABLE 6. Genes downregulated by the G648D mutation in Upc2p

CandidaDB name[a]	Common name[b]	orf19 no.[c]	GO annotation (molecular function)[d]	Fold decrease in expression					
				S2 vs S1		*UPC2*[S2-1] vs *UPC2*[S1-1]			
						Strain A		Strain B	
				Expt 1	Expt 2	Expt 1	Expt 2	Expt 1	Expt 2
COQ1		orf19.7478	*trans*-Hexaprenyltranstransferase activity	−2.8	−2.9	−1.5	−2.5	−2.4	−3.8
MAS2		orf19.6295	Mitochondrial processing peptidase activity	−1.6	−1.7	−1.6	−2.3	−2.7	−2.9
MET14	*MET14*	orf19.946	Adenylylsulfate kinase activity	−5.6	−6.9	−2.3	−2.9	−3.5	−3.7
MET3	*MET3*	orf19.5025	Sulfate adenylyltransferase (ATP) activity	−4.2	−3.8	−3.3	−4.0	−3.5	−5.9
PET127		orf19.2309	RNA binding	−1.7	−1.8	−2.0	−4.4	−7.7	−5.9
IPF11801		orf19.2798	Helicase activity	−1.6	−1.7	−2.3	−2.2	−2.0	−1.8
IPF14872		orf19.4133		−2.0	−1.8	−1.8	−3.5	−2.3	−4.0
IPF20118		orf19.3846		−3.8	−4.4	−3.6	−3.1	−1.7	−1.6
IPF525		orf19.7085		−2.4	−2.2	−1.7	−3.1	−1.7	−1.7
IPF6504		orf19.1544		−2.1	−2.0	−1.8	−1.5	−1.7	−1.6
IPF7334		orf19.3724		−2.3	−2.2	−1.6	−3.1	−1.8	−3.4

[a] Gene name from CandidaDB (http://genolist.pasteur.fr/CandidaDB/).
[b] Gene name from CGD (http://www.candidagenome.org/).
[c] orf19 nomenclature according to assembly 19.
[d] Gene Ontology (GO) (4) annotation found at CGD (http://www.candidagenome.org/).

specific for fluconazole, but not for other ergosterol biosynthesis inhibitors, it is unlikely that it contributes to the *UPC2*-dependent changes in susceptibility to terbinafine observed in the present study. Although we were unable to identify an SRE motif in the promoter region of *MDR1*, these data suggest that Upc2p may play a role in the regulation of *MDR1* expression.

As mentioned above, Sc*UPC2* has been implicated in the uptake of exogenous sterols. In *S. cerevisiae*, sterol uptake is mediated by an ABC transporter encoded by the *AUS1* gene

TABLE 7. Genes upregulated in both isolate S2 and transformants expressing the *UPC2*[S2-1] allele with SRE motifs in their promoter

CandidaDB name[a]	Common name[b]	orf19 no.[c]	SRE motifs[d]	Coordinates
DDR48	*DDR48*	orf19.4082	TGT**TCGTATA**ACT	−661 to −649
ECM3	*ECM3*	orf19.1563	ATG**TATACGA**TGA	−312 to −300
ERG1	*ERG1*	orf19.406	TCA**TCGTATA**TTT	−376 to −364
ERG11	*ERG11*	orf19.922	ATC**TATACGA**CGA	−489 to −477
			ATG**TCGTATA**TTC	−235 to −223
ERG24	*ERG24*	orf19.1598	CGT**TATACGA**CCG	−142 to −130
ERG26	*ERG26*	orf19.2909	CTC**TATACGA**CAA	−283 to −271
ERG27	*ERG27*	orf19.3240	GTT**TATACGA**TCG	−154 to −142
QRI8		orf19.7329	ATT**TCGTATA**TCT	−367 to −355
TEF41	*TEF4*	orf19.2652	TGC**TATACGA**TGG	−308 to −296
IPF18080		orf19.7357	CCC**TCGTATA**GCG	−910 to −898
IPF2903		orf19.7456	ATT**TATACGA**CAT	−278 to −266
IPF4059		orf19.1865	CGA**TCGTATA**TAC	−215 to −203
IPF6518		orf19.1691	TTC**TATACGA**CAG	−716 to −704
IPF7289	*UPC2*	orf19.391	AGC**TATACGA**GTT	−440 to −428
IPF7397		orf19.1800	TGA**TCGTATA**GAT	−566 to −554
IPF7432		orf19.6219	CAC**TATACGA**CGA	−353 to −341
IPF867	*SET3*	orf19.7221	TCG**TCGTATA**GAC	−544 to −532
			TGA**TCGTATA**GAG	−685 to −673
			GTC**TATACGA**CCA	−721 to −709
IPF8970		orf19.496	CAG**TCGTATA**CTG	−348 to −336
			TAT**TATACGA**TGG	−211 to −199

[a] Gene name from CandidaDB (http://genolist.pasteur.fr/CandidaDB/).
[b] Gene name from CGD (http://www.candidagenome.org/).
[c] orf19 nomenclature according to assembly 19.
[d] The SRE motifs are shown in bold.

(48). In *C. albicans*, Sc*AUS1* is homologous to a number of ABC transporters, including *CDR1* and *CDR2*. However, the actual mechanism of sterol uptake in *C. albicans* is unknown. By using microarray analysis, we found genes encoding ABC transporters to be upregulated by the *UPC2* gain-of-function mutation. In the resistant isolate S2, the ABC transporter gene *CDR12* was upregulated 1.5- to 3.0-fold, and in SC5314 transformants expressing the *UPC2*[S2-1] allele, the ABC transporter gene *CDR11* was upregulated 1.5- to 3.5-fold. According to the CGD, *CDR11* and *CDR12* are two different alleles of the gene *CDR11*. Because of the homology of *CDR11* to Sc*AUS1*, Cdr11p is a strong candidate for the transporter that mediates the uptake of exogenous sterols in *C. albicans*. This hypothesis is currently under investigation.

Recently, Znaidi et al. examined the Upc2p regulon using genome-wide location profiling (ChIP-on-chip) (50). In order to perform chromatin immunoprecipitation, a triple hemagglutinin epitope was introduced at the C terminus of Upc2p. This also resulted in a gain-of-function mutation. Using this approach, they successfully identified 202 genes whose promoters are bound by Upc2p. Among the 32 upregulated genes in the core set of Upc2p target genes identified in our study (as well as *ERG11*), 10 were also among those identified by Znaidi et al. as being direct Upc2p targets. Among these were *ERG11*, *MDR1*, *ECM3*, *ERG1*, *ERG24*, *NCP1*, *SET3*, and *UPC2* itself. Limitations of the microarrays used in our expression analysis and the location analysis of Znaidi et al. may have resulted in the failure of other Upc2p target genes to be identified in one study or the other. It is also possible that many of the genes implicated in location analysis require more than the activation of Upc2p for their expression.

The identification of a mutation in *UPC2* that mediates fluconazole resistance in a clinical *C. albicans* isolate closes another gap in our understanding of the molecular mechanisms of drug resistance in this human fungal pathogen. In addition to point mutations in *ERG11* that result in reduced affinity of azole drugs to their target enzyme, the genetic basis of alterations in gene expression that are linked to fluconazole

resistance has now also been unraveled. The transcription factors controlling the expression of efflux pumps and ergosterol biosynthesis genes have recently been identified (7, 27, 31, 43). In all cases examined so far, overexpression of the ABC transporters *CDR1* and *CDR2* is caused by gain-of-function mutations in the transcription factor Tac1p (5, 6, 49), overexpression of the major facilitator *MDR1* is caused by gain-of-function mutations in Mrr1p (31), and as shown here, overexpression of *ERG11* is caused by a gain-of-function mutation in Upc2p. All three transcription factors belong to the zinc cluster transcription factor family that is found only in fungi (28). It is likely that additional mutations in these transcription factors will be found in other fluconazole-resistant *C. albicans* isolates, underscoring their key role in the development of drug resistance.

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