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Contribution of Clinically Derived Mutations in *ERG11* to Azole Resistance in *Candida albicans*

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In *Candida albicans*, the *ERG11* gene encodes lanosterol demethylase, the target of the azole antifungals. Mutations in *ERG11* that result in an amino acid substitution alter the abilities of the azoles to bind to and inhibit Erg11, resulting in resistance. Although *ERG11* mutations have been observed in clinical isolates, the specific contributions of individual *ERG11* mutations to azole resistance in *C. albicans* have not been widely explored. We sequenced *ERG11* in 63 fluconazole (FLC)-resistant clinical isolates. Fifty-five isolates carried at least one mutation in *ERG11*, and we observed 26 distinct positions in which amino acid substitutions occurred. We mapped the 26 distinct variant positions in these alleles to four regions in the predicted structure for Erg11, including its predicted catalytic site, extended fungus-specific external loop, proximal surface, and proximal surface-to-heme region. In total, 31 distinct *ERG11* alleles were recovered, with 10 *ERG11* alleles containing a single amino acid substitution. We then characterized 19 distinct *ERG11* alleles by introducing them into the wild-type azole-susceptible *C. albicans* SC5314 strain and testing them for susceptibilities to FLC, itraconazole (ITC), and voriconazole (VRC). The strains that were homozygous for the single amino acid substitutions Y132F, K143R, F145L, S405F, D446E, G448E, F449V, G450E, and G464S had a ≥4-fold increase in FLC MIC. The strains that were homozygous for several double amino acid substitutions had decreased azole susceptibilities beyond those conferred by any single amino acid substitution. These findings indicate that mutations in *ERG11* are prevalent among azole-resistant clinical isolates and that most mutations result in appreciable changes in FLC and VRC susceptibilities.

Candida albicans is the most prevalent cause of fungal disease (1). Clinical manifestations of infections with *Candida* species can range from superficial mucosal infections to deep organ involvement usually resulting from hematogenous spread of infection. Despite the significant progress that has been made in the management of patients with fungal infections, the emergence of antifungal-resistant isolates creates a significant problem with regard to antifungal prophylaxis and empirical treatment strategies (2, 3). The azole antifungal class has been the so-called "work horse" of antifungal pharmacotherapy for the past 30 years, defined by its efficacy against *Candida* species and paucity of side effects. As the only oral option available for systemic antifungal treatment, the azoles are the most suitable option for the long treatment periods sometimes required for antifungal prophylaxis and therapy. As the azoles are fungistatic against *Candida* species, lengthy and repeated treatment courses have resulted in azole-resistant clinical isolates, resulting in treatment failure.

Sterols are essential components that function to maintain fluidity in eukaryotic membranes. The azole class of antifungals inhibits ergosterol biosynthesis and allows for the accumulation of toxic methylated sterol precursors (4). The primary sterol in the fungal cell membrane is ergosterol, and Cyp51 in *C. albicans* is a critical part of this biosynthetic process. Cyp51 in *C. albicans* (CaCyp51) catalyzes a three-step reaction that ultimately results in the demethylation of lanosterol. Each step requires one molecule of oxygen and NADPH. The azoles inhibit lanosterol demethylase by binding the nucleophilic N-4 atom of the azole ring to the heme iron at its sixth coordinate position (5). The normal substrate for this enzyme is lanosterol, and azole derivatives sit in the same binding pocket. Biochemical analysis shows that all azoles bind selectively to CaCyp51; however, the K_d (dissociation constant) values show a 2-fold to 4-fold lower affinity to fluconazole than that to itraconazole or voriconazole (5).

In *C. albicans*, the modulation of the *ERG11* gene in the ergosterol biosynthetic pathway and the alteration of the Erg11 protein targeted by azole antifungals have been shown to contribute to azole resistance. The overexpression of *ERG11* transcripts, either by gain-of-function mutations (GOF) in the transcriptional regulator, Upc2, or increased chromosome 5 copy number (on which *ERG11* resides), result in reduced azole susceptibility (6–8). Mutations in the Erg11 protein mediating lanosterol demethylation have been shown to alter the ability of azole antifungals to bind to and inhibit its activity and to result in enhanced resistance to this class of antifungal agents (9–11). Previous reports of mutations in *ERG11* have defined three hot spot regions corresponding to amino acids 105 to 165, 266 to 287, and 405 to 488, which are particularly permissive to amino acid substitutions (12). In order to show that *ERG11* mutations can contribute to azole resistance, investigators have used several approaches, including heterologous expression of mutant *ERG11* alleles in other microbial spe-

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TABLE 1 *C. albicans* strains carrying mutant *ERG11* alleles used in this study

C. albicans strain	Relevant characteristic(s) or genotype	Source or reference
SC5314	*ERG11-1/ERG11-2*	ATCC
Clinical isolate		
10-72	Azole resistant	University of Iowa
Constructed laboratory strains[a]		
ERG11WT A1A50A	$ERG11^{WT}$::*FRT*/$ERG11^{WT}$::*FRT*	This study
ERG11WT B37A30A	$ERG11^{WT}$::*FRT*/$ERG11^{WT}$::*FRT*	This study
5A2A43A	$ERG11^{Q21L}$::*FRT*/$ERG11^{Q21L}$::*FRT*	This study
5B6A19A	$ERG11^{Q21L}$::*FRT*/$ERG11^{Q21L}$::*FRT*	This study
20E1II1G1	$ERG11^{Y132F}$::*FRT*/$ERG11^{Y132F}$::*FRT*	This study
20B5A14A	$ERG11^{Y132F}$::*FRT*/$ERG11^{Y132F}$::*FRT*	This study
10C1B1M1	$ERG11^{K143R}$::*FRT*/$ERG11^{K143R}$::*FRT*	This study
10B1A32A	$ERG11^{K143R}$::*FRT*/$ERG11^{K143R}$::*FRT*	This study
2A1A18A	$ERG11^{F145L}$::*FRT*/$ERG11^{F145L}$::*FRT*	This study
2B1A51A	$ERG11^{F145L}$::*FRT*/$ERG11^{F145L}$::*FRT*	This study
21C1M1A1	$ERG11^{S405F}$::*FRT*/$ERG11^{S405F}$::*FRT*	This study
21B12A61B	$ERG11^{S405F}$::*FRT*/$ERG11^{S405F}$::*FRT*	This study
22AABA56A	$ERG11^{D446E}$::*FRT*/$ERG11^{D446E}$::*FRT*	This study
22B12A58A	$ERG11^{D446E}$::*FRT*/$ERG11^{D446E}$::*FRT*	This study
20NA11A57A	$ERG11^{G448E}$::*FRT*/$ERG11^{G448E}$::*FRT*	This study
20NB16A10A	$ERG11^{G448E}$::*FRT*/$ERG11^{G448E}$::*FRT*	This study
7A5A5A	$ERG11^{F499V}$::*FRT*/$ERG11^{F499V}$::*FRT*	This study
7B4A29A	$ERG11^{F449V}$::*FRT*/$ERG11^{F449V}$::*FRT*	This study
15A3A108A	$ERG11^{G450E}$::*FRT*/$ERG11^{G450E}$::*FRT*	This study
16A14A47A	$ERG11^{G450E}$::*FRT*/$ERG11^{G450E}$::*FRT*	This study
19A1A1C1	$ERG11^{G464S}$::*FRT*/$ERG11^{G464S}$::*FRT*	This study
19B1A71A	$ERG11^{G464S}$::*FRT*/$ERG11^{G464S}$::*FRT*	This study
6A1A47A	$ERG11^{K143R,E266D}$::*FRT*/$ERG11^{K143R,E266D}$::*FRT*	This study
6B18A101A	$ERG11^{K143R,E266D}$::*FRT*/$ERG11^{K143R,E266D}$::*FRT*	This study
27A5A33A	$ERG11^{Y132F,F145L}$::*FRT*/$ERG11^{Y132F,F145L}$::*FRT*	This study
27B7A63A	$ERG11^{Y132F,F145L}$::*FRT*/$ERG11^{Y132F,F145L}$::*FRT*	This study
8A4A1A	$ERG11^{G450E,I483V}$::*FRT*/$ERG11^{G450E,I483V}$::*FRT*	This study
8B4A47A	$ERG11^{G450E,I483V}$::*FRT*/$ERG11^{G450E,I483V}$::*FRT*	This study
9A14A21	$ERG11^{Y132F,K143R}$::*FRT*/$ERG11^{Y132F,K143R}$::*FRT*	This study
9B4B34A	$ERG11^{Y132F,K143R}$::*FRT*/$ERG11^{Y132F,K143R}$::*FRT*	This study
7NA35A40A	$ERG11^{F145L,E266D}$::*FRT*/$ERG11^{F145L,E266D}$::*FRT*	This study
7NB44A56A	$ERG11^{F145L,E266D}$::*FRT*/$ERG11^{F145L,E266D}$::*FRT*	This study
13A1A57A	$ERG11^{D278N,G464S}$::*FRT*/$ERG11^{D278N,G464S}$::*FRT*	This study
13B6A16A	$ERG11^{D278N,G464S}$::*FRT*/$ERG11^{D278N,G464S}$::*FRT*	This study
29NA24A23A	$ERG11^{E266D,G464S}$::*FRT*/$ERG11^{E266D,G464S}$::*FRT*	This study
29NB30A22A	$ERG11^{E266D,G464S}$::*FRT*/$ERG11^{E266D,G464S}$::*FRT*	This study
30A5A53A	$ERG11^{M258L,G464S}$::*FRT*/$ERG11^{M258L,G464S}$::*FRT*	This study
30B5A57A	$ERG11^{M258L,G464S}$::*FRT*/$ERG11^{M258L,G464S}$::*FRT*	This study
8OA31A17A	$ERG11^{G307S,G450E}$::*FRT*/$ERG11^{G307S,G450E}$::*FRT*	This study
8OB37A4A	$ERG11^{G307S,G450E}$::*FRT*/$ERG11^{G307S,G450E}$::*FRT*	This study

[a] All laboratory strains have SC5314 as background.

cies (including *Saccharomyces cerevisiae* and *Pichia pastoris*), enzyme inhibition with fluconazole (FLC) in cell extracts, and biochemical analysis (10, 11, 13, 14). While a number of different amino acid substitutions have been associated with azole resistance (13), the majority of the previously analyzed mutations have not been studied in *C. albicans*.

Advances in the sequencing of the *C. albicans* genome and transformation of this pathogen have now allowed the study of *ERG11* variations in the pathogen itself. For this, we examined the prevalence and variance of *ERG11* mutations in a group of 63 characterized clinical *C. albicans* isolates with reduced FLC susceptibilities. We then expressed a select group of these *ERG11* mutant alleles in an azole-susceptible *C. albicans* strain to determine the relative contributions of the individual *ERG11* mutations to azole susceptibility, and we mapped the critical positions of variation on the predicted structure of CaErg11 (Cyp51).

MATERIALS AND METHODS

Strains and growth conditions. *C. albicans* strains were cultured on YPD (1% yeast extract, 2% peptone, and 2% dextrose) agar plates at 30°C and stored as frozen stock in 40% glycerol at −80°C (Table 1). YPD liquid medium was used for the routine growth of the strains. Nourseothricin (200 μg/ml or 25 μg/ml) was added to YPD agar plates to select strains containing the *SAT1* flipper cassette (15). For plasmid construction and propagation, One Shot *Escherichia coli* TOP10 chemically competent cells (Invitrogen, Carlsbad, CA) were used and grown in Luria-Bertani (LB)

TABLE 2 *ERG11* primers used in this study

Primer name by purpose	Sequence[a]
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	
T7	5′-TAATACGACTCACTATAGGG-3′
*ERG11*seqB	5′-TATTTTCACTGCTTCAAGATCT-3′
*ERG11*seqC	5′-CCAAAAGGTCATTATGTTTTAG-3′
M13R	5′-CAGGAAACAGCTATGACC-3′
*ERG11*seqE	5′-CATTTAGGTGAAAAACCTCATT-3′
*ERG11*seqF	5′-TACTCCAGTTTTCGGTAAAGGG-3′

[a] Underlined sequences reflect the introduction of a restriction site sequence.

broth or on LB agar plates supplemented with 50 μg/ml kanamycin (Fisher BioReagents, Fair Lawn, NJ) or 100 μg/ml ampicillin (Sigma).

Plasmid construction for allele sequencing. The *CaERG11* coding sequences were amplified by PCR (*Pfu* DNA polymerase; Stratagene) from *C. albicans* genomic DNA using the primers *ERG11*-A and *ERG11*-E (Table 2). The products were cloned into pCR-BluntII-TOPO using a Zero Blunt TOPO PCR cloning kit (Invitrogen) and transferred into *E. coli* TOP10 cells, with selection on LB agar plates containing 50 μg/ml kanamycin. Plasmid DNA was purified (QIAprep; Qiagen, Germantown, MD) and sequenced on an ABI 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 frames (ORFs) whose predicted translations indicated an amino acid substitution were digested with the restriction enzymes ApaI and XhoI, which excised the full-length ORF from the plasmid, and these *ERG11* coding sequences were cloned upstream of the *SAT1* flipper cassette into the ApaI and XhoI sites of plasmid pSFS2 (15). The *ERG11* downstream segments were amplified with *Ex Taq* (TaKaRa) using primers *ERG11*-C and *ERG11*-D and cloned downstream of the *SAT1* flipper cassette in pSFS2 using the NotI and SacII sites. This process generated plasmids (and their substitutions) pERG11-2 (F145L), pERG11-5 (Q21L), pERG11-6 (K143R+E266D), pERG11-7 (F449V), pERG11-7N (F145L+E266D), pERG11-8 (G450E+I483V), pERG11-8O (G307S+G450E), pERG11-9 (Y132F+K143R), pERG11-10 (K143R), pERG11-13 (D278N+G464S), pERG11-15/16 (G450E), pERG11-19 (G464S), pERG11-20 (Y132F), pERG11-20N (G448E), pERG11-21 (S405F), pERG11-22 (D446E), pERG11-27 (Y132F+F145L), pERG11-29N (E266D+G464S), and pERG11-30 (M258L+G464S).

Construction of strains carrying specific *ERG11* alleles. *C. albicans* strain SC5314 was transformed by electroporation with gel-purified inserts from each pSF2-derived plasmid that contained the *SAT1* flipper disruption cassette developed by Reuss et al. (15). In these, the *SAT1* selectable marker that confers resistance to nourseothricin and the *FLP* flipper recombinase gene are both flanked by flipper recombinase target (FRT) sites, allowing for the direct selection of nourseothricin-resistant transformants carrying only the *ERG11* allele with a downstream FRT left in the *ERG11* locus, as previously described (15). The integration of the constructs was confirmed by Southern hybridization.

Azole susceptibility testing. MICs were obtained by using a modified CLSI protocol outlined in document M27-A3 (16), using RPMI medium. Overnight cultures grown at 30°C were streaked onto Sabouraud's agar and then grown for 24 h at 30°C. Individual colonies were suspended in sterile water until an optical density at 600 nm of 0.1 was reached. The working colony concentration was made by making a 1:50 dilution and a 1:20 dilution sequentially in medium. One hundred microliters from the working stock was used to inoculate a series of azole/RPMI medium dilutions, with the highest being 64 μg/ml for fluconazole. Similar procedures were used for the voriconazole and itraconazole dilutions; however, the highest concentration used for these agents was 8 μg/ml. The cultures were incubated at 35°C for 48 h, and the MICs were recorded.

Homology modeling. The construction of the Cyp51 homology model was done according to the single-template approach outlined by Baudry, Rupasinghe, and Schuler (17) and Rupasinghe and Schuler (18) using functions within MOE (version 2011; Chemical Computing Group, Inc., Montreal, Canada). Based on the sequence identities returned by the BLOSUM62 scoring matrix (19) within the ALIGN function in MOE for the wild-type *C. albicans* sequence (GenBank accession no. XM_711668), *S. cerevisiae* Cyp51 (Genpept accession no. 4LXJ_A), and other structurally defined Cyp51 proteins, the lanosterol-bound *S. cerevisiae* Cyp51 (PDB 4LKJ [20]), sharing 66% amino acid identity, was chosen as the template, with no replacements in the variable regions. A second *C. albicans* allele (GenBank accession no. XM_711729) characterized in this study as wild type contains two variant positions (D116E and K128T) that do not affect azole resistance levels.

The predicted structures for the wild-type *C. albicans* Cyp51 were constructed with this single substrate-bound template using the homology function in MOE to generate 10 coarsely energy-minimized models. The model generated with the best packing score was energy minimized using the CHARMM22 force field (21) in MOE. The predicted structures were then inspected to ensure that all major P450 structural motifs (FG loop, I-helix, and the substrate access channel) were intact. Ramachandran plots were used to evaluate any torsional outliers in the final energy-minimized model, and the models generated with torsional outliers within a SAG1-related sequence (SRS) site or near the active site cavity were scrapped.

The DOCK function in MOE was used to predict the binding mode of the inhibitor FLC in the Cyp51 catalytic site identified using the Site Finder function in MOE. Simulations were run using the MMFF94x force field to optimize the binding configuration by scoring spatial contacts and electrostatic interactions (22, 23). With 100 configurations set as an upper limit for the docking, the populated list of configurations was searched for docking modes that placed these inhibitors within close proximity to the heme-bound oxygen in orientations consistent with azole inhibition. Promising configurations were then taken through another round of energy minimization under the MMFF94x force field, with a flexible protein backbone and ligand, rigid heme, and a target energy gradient of 0.1 kcal/mol · Å.

The interaction energy between each potential inhibitor and Cyp51 was calculated as the difference between the total potential energy of the minimized complex and the sum of the individual protein and ligand components of the minimized complex. The potential energy function contains the sum of the ligand/protein internal energy, van der Waals, and electrostatic energy terms. The conformer with the lowest calculated interaction energy was selected as the most probable binding interaction.

Nucleotide sequence accession numbers. The coding sequences of the *ERG11* alleles described in this study have been deposited in GenBank under the accession numbers KM875712, KM875713, KM875714, KM875715, KM875716, KM875717, KM875718, KM875719, KM875720, KM875721, KM875722, KM875723, KM875724, KM875725, KM875726, KM875727, KM875728, KM875729, and KM881482.

RESULTS

Many clinical isolates with reduced fluconazole susceptibilities carry mutations in *ERG11*. Of the 63 isolates that were determined to be resistant to fluconazole (i.e., MIC, ≥8 μg/ml), 55 carried a mutation in *ERG11* that led to at least one amino acid substitution. Although silent mutations were observed in the *ERG11* alleles tested (data not shown), we recovered 26 distinct

positions in which mutations occurred that resulted in an amino acid substitution either alone or in combination with other mutations (Table 3 and Fig. 1). With the exception of isolate 16, which harbored a Q21R substitution in only one *ERG11* allele, other isolates were homozygous for mutations in the *ERG11* allele. Nine of these substitutions, due to either their position (Q21R, M258L, L403F, and I483V) or the specific amino acid substitution (A114V, D446E, Y447S, F449I, and I471M), have not been described in previous reports. Among the isolates that carry amino acid substitutions in the Erg11 protein, the number of substitutions varied between the isolates and ranged between 1 ($n = 28$) and 4 ($n = 3$), with most isolates in this collection carrying a single amino acid substitution. In total, 31 unique *ERG11* alleles were recovered from sequence analysis (Table 3), with the most common polymorphisms detected at positions E266 ($n = 10$), Y132 ($n = 9$), G464 ($n = 8$), and K143 ($n = 7$).

Single mutations in *ERG11* contribute to azole resistance in *C. albicans*. In order to assess the contribution of each individual mutant *ERG11* allele to azole antifungal resistance, we expressed each *ERG11* allele homozygously in each constructed strain. Each strain was constructed in duplicate, and its susceptibilities against fluconazole, itraconazole, and voriconazole were tested and compared to those of the wild-type susceptible parent SC5314 strain at 48 h (Fig. 2). Two independent strains were constructed to carry two *ERG11* wild-type alleles, which did not show a change in MIC compared to that of the parent strain for any of the azoles tested. We initially examined the effects of 10 mutant *ERG11* alleles containing a single amino acid substitution in their predicted protein sequence, including Q21L, Y132F, K134R, F145L, S405F, D446E, G448E, F449V, G450E, and G464S. With the exception of the amino acid substitution Q21L, most *ERG11* mutations resulted in decreased susceptibility to fluconazole compared to that of SC5314 (Fig. 2). Strains homozygously expressing K143R resulted in the strongest decrease in fluconazole susceptibility. The strains that were homozygous for alleles containing single amino acid substitutions Y132F, F145L, S405F, D446E, D448E, F449V, G464S, or G450E had a ≥4-fold increase in their fluconazole MIC. No single amino acid substitution affected itraconazole or voriconazole MICs more than 2-fold. These data suggest that structural differences between the individual azoles affect their activities against specific mutant *ERG11* alleles.

Multiple mutations in *ERG11* can result in decreased azole susceptibility. Previous observations showed that combinations of *ERG11* mutations can lead to considerable increases in the MICs to fluconazole (14, 24). To examine the effects of multiple mutations on azole susceptibility, we selected a group of clinically occurring *ERG11* alleles that carried two amino acid substitutions, with at least one substitution having been characterized alone (Fig. 2). Of those analyzed, the allele with the K143R and Y132F substitutions showed the strongest characterized combination effect, with 32-fold and 4-fold increased FLC and VRC MICs, respectively, over those observed for the azole-susceptible SC5314 strain. Interestingly, this combination did not affect ITC susceptibility. Other notable increases in the FLC MICs were detected for the Y132F+F145L and G307S+G450E combinations, with both increasing their FLC MICs by 16-fold over that observed for the SC5314 strain. Unlike all other *ERG11* alleles we characterized, the Y132F+F145L combination also significantly increased ITC and VRC MICs by 4-fold and 16-fold, respectively.

Among the other combinations of polymorphisms present in our collection, the I483V+G450E combination increased the FLC MIC by 8-fold over that of the SC5314 strain and by 2-fold over that observed for the G450E allele. This combination did not significantly change either the ITC or VRC MIC. Similarly, combinations of M258L or D278N with G464S increased their FLC MICs by 2-fold and 4-fold, respectively, over that observed for the G464S allele.

Amino acid substitution E266D does not contribute to azole resistance. The E266D substitution was the most prevalent polymorphism detected by sequence analysis, and it occurred only in combination with other amino acid substitutions. To investigate the contribution of E266D to azole resistance, we compared the susceptibilities of *ERG11* alleles carrying one amino acid substitution alone to an allele containing an identical amino acid substitution combined with E266D. In our collection, the K143R, F145L, and G464S substitutions all occurred as single mutations and in combination with the E266D substitution. In all three combinations, the E266D substitution did not confer any additional effect on azole susceptibility beyond what was observed with the single amino acid substitution.

Molecular mapping of Cyp51 variant positions. To better understand the positions of the variations enhancing FLC resistance, all were mapped on a predicted structure for ligand-free wild-type Cyp51 (GenBank accession no. XM_711668), derived using the modeling techniques described by Baudry, Rupasinghe, and Schuler (17) and Rupasinghe and Schuler (18), with lanosterol-bound *S. cerevisiae* Cyp51 (PDB 4LXJ [20]) as the backbone. As shown in Fig. 3A, single amino acid variations affecting resistance to one or more of these azole inhibitors occur in the predicted catalytic site (Y132F and S405F), the extended fungus-specific external loop (D446E, G448E, F449V, and G450E), on the proximal surface with potential loop interactions (F145L), and between the proximal surface and heme (K143R and G464S). As shown in Fig. 3B, double amino acid variations affecting resistance to one or more azole inhibitors exist, with additional changes occurring in the catalytic site (G307S and I483V) and on the surface (M258L, E266D, and D278N). A graph of the resistance levels in these mutants relative to the positions of these variations shown in Fig. 2 allows several conclusions to be made. First, the combination of Y132F+F145L variations mapping to the catalytic site and proximal surface maintain the resistance level for FLC as in the K143R single variant while moderately increasing the resistance level for ITC and dramatically increasing the resistance level for VRC. Second, the combination of Y132F+K143R variations mapping to the catalytic site and region below the heme dramatically increases the resistance level for FLC compared to that of the K143R single variant while moderately increasing the resistance level for VRC. Third, the combination of G307S+G450E mapping to the catalytic site and external loop dramatically increases the resistance level to FLC compared to that of the G450E single variant. To a lesser extent, the combination of I483V+G450E variations mapping to the catalytic site and external loop also significantly increases the resistance level for FLC compared to that of the G450E single variant while having no effect on the resistance levels of the other azoles. Fourth, the combination of D278N+G464S mapping to the surface and proximal regions significantly increases the resistance level for VRC and dramatically increases the resistance level for FLC compared to that with the G464S single variant. To a lesser extent, the combination of the M258L+G464S variations mapping to the surface and proximal region also increases the

TABLE 3 Occurrence of Erg11 amino acid (aa) substitutions in the predicted translated sequence in fluconazole-resistant clinical *ERG11*-overexpressing isolates

Erg11 aa substitution(s) (*ERG11* mutation)	ID[a]	Zygosity	FLC MIC (μg/ml)	Upc2 aa substitution[b]	Other resistance mechanism(s)[b]
None	36		64		↑ *ERG11*, *CDR1*, *CDR2*
	37		64		↑ *ERG11*, *CDR1*, *CDR2*
	53		>256		↑ *ERG11*
	55		>256	G648S	↑ *ERG11*
	56		>256	G648S	↑ *ERG11*, *CDR1*, *CDR2*
	57		>256	G648S	↑ *ERG11*, *CDR1*, *CDR2*
	58		>256	G648S	↑ *ERG11*, *CDR1*, *CDR2*
	60		>256		↑ *ERG11*
Q21R (A62G)	16	Heterozygous	16		↑ *MDR1*
Y132F (A395T)	48	Homozygous	256		↑ *ERG11*, *CDR1*, *CDR2*
K143R (A428G)	13	Homozygous	16	A643T	↑ *ERG11*, *CDR1*, *CDR2*
	12	Homozygous	16		↑ *ERG11*, *CDR1*, *CDR2*
	14	Homozygous	16		↑ *ERG11*, *CDR1*, *CDR2*
F145L (T435G)	22	Homozygous	32		↑ *ERG11*
S405F (C1214T)	18	Homozygous	32		↑ *ERG11*, *CDR1*, *CDR2*
	19	Homozygous	32		↑ *ERG11*, *CDR1*, *CDR2*
	20	Homozygous	32		↑ *ERG11*, *CDR1*, *CDR2*
	41	Homozygous	128		↑ *ERG11*, *MDR1*
D446E (T1338A)	25	Homozygous	32	G648S[c]	↑ *ERG11*, *MDR1*
	35	Homozygous	64	G648S[c]	↑ *ERG11*
	44	Homozygous	128		↑ *ERG11*, *CDR1*, *CDR2*, *MDR1*
G448E (G1341A)	31	Homozygous	64		↑ *ERG11*, *CDR1*, *CDR2*
	65	Homozygous	>256	G648S[c]	↑ *ERG11*, *CDR1*, *CDR2*
	66	Homozygous	>256		↑ *CDR1*, *CDR2*
	67	Homozygous	>256	G648S[c]	↑ *ERG11*, *CDR1*, *CDR2*
	69	Homozygous	>256	G648S[c]	↑ *ERG11*, *CDR1*, *CDR2*
F449V (T1345G)	40	Homozygous	128		↑ *ERG11*, *CDR1*, *CDR2*
G450E (G1349A)	24	Homozygous	32		↑ *CDR1*, *CDR2*, *MDR1*
	34	Homozygous	64	Y642F	↑ *CDR1*, *CDR2*, *MDR1*
	63	Homozygous	>256		↑ *ERG11*, *CDR1*, *CDR2*, *MDR1*
	64	Homozygous	>256		↑ *ERG11*, *CDR1*, *CDR2*, *MDR1*
G464S (G1390A)	32	Homozygous	64		↑ *CDR1*, *CDR2*, *MDR1*
	38	Homozygous	64	G648D[c]	↑ *ERG11*, *CDR1*, *CDR2*
	39	Homozygous	64		↑ *ERG11*, *CDR1*, *CDR2*
	42	Homozygous	128		↑ *ERG11*, *CDR1*, *CDR2*
	52	Homozygous	256	G648D[c]	↑ *ERG11*
A114S (G340T), Y257H (T769C)	17	Homozygous	32		↑ *CDR1*
F126L (C378A), Y132F (A395T)	30	Homozygous	64	A643V[c]	↑ *ERG11*
Y132F (A395T), K143R (A428G)	50	Homozygous	256		↑ *CDR1*, *CDR2*
	61	Homozygous	>256		↑ *CDR1*, *CDR2*
Y132F (A395T), F145L (T435G)	29	Homozygous	64		↑ *CDR1*, *CDR2*
K143R (A428G), E266D (A798C)	10	Homozygous	16		↑ *ERG11*, *CDR1*
F145L (T435G), E266D (A798C)	33	Homozygous	64	Y642F	↑ *CDR1*, *CDR2*
M258L (A772T), G464S (G1390A)	70	Homozygous	>256	A646V[c]	↑ *ERG11*
E266D (A798C), G464S (G1390A)	45	Homozygous	128	A646V[c]	↑ *ERG11*, *CDR1*, *CDR2*
	15	Homozygous	16	W478C[c]	↑ *ERG11*, *CDR1*, *CDR2*
	27	Homozygous	64	W478C[c]	↑ *ERG11*, *CDR1*, *CDR2*
E266D (A798C), V488I (G1312A)	11	Homozygous	16		↑ *ERG11*, *CDR1*, *CDR2*
	28	Homozygous	64	W478C[c]	↑ *ERG11*, *CDR1*, *CDR2*

(Continued on following page)

TABLE 3 (Continued)

Erg11 aa substitution(s) (*ERG11* mutation)	ID[a]	Zygosity	FLC MIC (μg/ml)	Upc2 aa substitution[b]	Other resistance mechanism(s)[b]
D278N (G832A), G464S (G1390A)	43	Homozygous	128		↑ *CDR1*
	51	Homozygous	256		↑ *ERG11*, *CDR1*, *CDR2*
	62	Homozygous	>256		↑ *CDR1*, *CDR2*
G307S (G919A), G450E (G1349A)	26	Homozygous	32		
	54	Homozygous	>256		↑ *ERG11*, *MDR1*
G450E (G1349A), I483V (A1309G)	23	Homozygous	32		↑ *CDR1*, *CDR2*, *MDR1*
A114V (C341T), E226D (A798C), H283R (A848G)	21	Homozygous	32	G648D[c]	↑ *ERG11*, *CDR1*, *CDR2*
Y132F (A395T), T229A (A685G), F449L (T1345A)	59	Homozygous	>256	G648D[c]	↑ *ERG11*, *CDR1*, *CDR2*
Y132F (A395T), V437I (G1309A), F449L (T1345C)	71	Homozygous	>256	G648D[c]	↑ *ERG11*, *CDR1*, *CDR2*
G307S (G919A), L403F (A1209T), G448R (G1342C)	46	Homozygous	128		
G307S (G919A), V437I (G1309A), Y447S (A1340C)	49	Homozygous	256		↑ *ERG11*, *CDR1*, *CDR2*
A114V (C341T), D153E (T459G), E266D (A798C), G450E (G1349A)	72	Homozygous	>256		↑ *CDR1*, *CDR2*
A114V (C341T), Y132F (A395T), E266D (A798C), V437I (G1309A)	47	Homozygous	128	G648D[c]	↑ *ERG11*, *CDR1*, *CDR2*
Y132F (A395T), E266D (A798C), I471M (T1413G), I483V (A1309G)	68	Homozygous	>256	T273A, A643V	↑ *ERG11*, *CDR1*, *CDR2*, *MDR1*

[a] ID, identification.
[b] Data were previously published by Flowers et al (6).
[c] Amino acid substitution recovered in one of two *UPC2* alleles.

resistance level to FLC while having little effect on the resistance levels to the other azoles. Finally, the E266D variation occurring in a number of double variants and mapping to the surface has no effect on resistance levels.

Low-throughput docking in the predicted wild-type Cyp51 catalytic site using the DOCK function in MOE with fluconazole (Fig. 4) suggests that it binds with one of its two azole rings coordinated with the heme. Based on this predicted binding mode,

Amino Acid Position

Species	21	114	126	132	143	145	153	229	257	258	266	278	283	307	403	405	437	446	447	448	449	450	464	471	483	488
Candida albicans	Q	A	F	Y	K	F	D	T	Y	M	E	D	H	G	L	S	V	D	Y	G	F	G	G	I	I	V
Candida tropicalis	Q	A	F	Y	K	F	D	T	Y	M	E	D	N	G	L	S	V	D	Y	G	F	G	G	I	I	I
Saccharomyces cerevisiae	Q	A	F	Y	K	F	E	T	Y	M	K	D	N	G	L	S	.	D	Y	G	F	G	G	I	L	I
Candida glabrata	Q	A	F	Y	K	F	E	T	Y	M	E	D	N	G	L	S	.	D	Y	G	F	G	G	I	L	I
Candida krusei	.	A	F	Y	K	F	E	T	Y	L	N	D	N	G	L	S	I	D	Y	G	F	G	G	T	.	.
Human (*Homo sapiens*)	C	A	F	Y	K	M	A	S	F	Y	Q	L	D	A	C	S	.	.	.	.	.	.	G	I	I	L
Mycobacterium tuberculosis	.	Q	F	F	N	L	E	D	V	A	A	D	A	A	A	S	.	.	.	.	.	.	G	V	I	L
Wheat (*Triticum aestivum*)	.	Q	F	F	T	F	N	L	F	A	A	L	D	A	A	S	.	.	.	.	.	.	G	L	I	L
Observed a.a. substitutions	R	V	L	F	R	L	E	A	H	L	D	N	R	S	F	F	I	E	S	E	I	E	S	M	V	I
		S		H	E						Q	E	D			P		N	G	R	L	R		T		G
																		G	H	V	S	V		V		
																					V					
																					Y					
Reference	This study	35	36, 37	37, 38	33, 38–40	38, 40	12, 40	36, 37	32, 38, 41	This study	12, 36, 38, 41, 42	43	38	37, 38	This Study	14, 36–38	14, 36, 37, 39	35, 37 this study	13, 35, this study	42, 44	36–39, 41	35–38, 44	14, 34, 36, 43	43, 45, this study	This study	38, 41, 44

FIG 1 Observed amino acid substitutions in Erg11 in *C. albicans* compared to those in other medically important *Candida* species and organisms in other kingdoms. The alignment was generated by using the UniProt alignment function (www.uniprot.org). The amino acid numbering is based on the *C. albicans* sequence. The conserved amino acids are highlighted in blue. The amino acid substitutions in red type are those observed in this study. The substitutions in black type have been noted in the literature (13). The following GenBank accession numbers were used: *C. albicans*, P1613; *Candida tropicalis*, P14263; *S. cerevisiae*, P1614; *Candida glabrata*, P50859; *C. krusei*, Q02315; human (*Homo sapiens*), Q16850; *Mycobacterium tuberculosis*, P0A512; *Triticum aestivum*, P93596. a.a., amino acid.

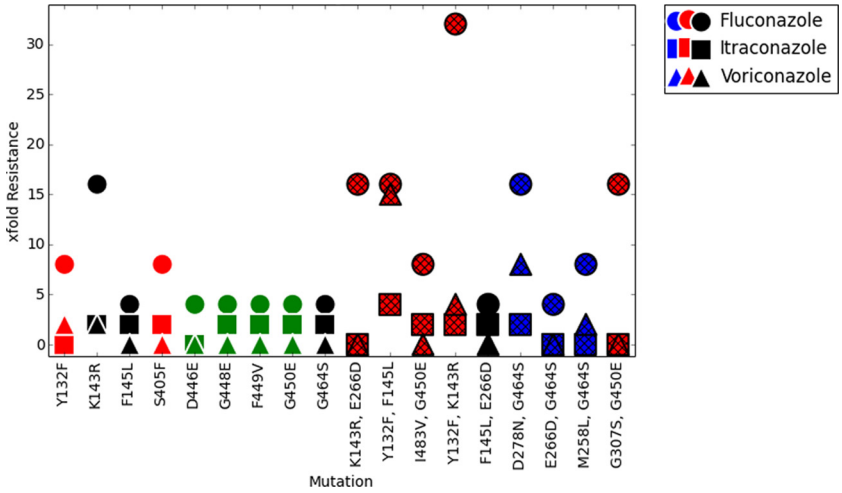

FIG 2 Azole resistance levels relative to amino acid variation(s) in predicted Cyp51 structure. The resistance levels of single and double mutants to FLC, ITC, and VRC are graphed, with red designating variations in or close to catalytic site residues, black designating variations in residues on the proximal side of the heme, green designating fungus-specific external loop residues, and blue designating surface residues. The strains that were constructed to carry two copies of the *ERG11* wild-type allele showed no appreciable change in azole MICs over those of SC5314 (data not shown).

G307 and Y132 are within interaction distance of FLC, and their changes in the G307S and Y132F variants are predicted to interfere with FLC binding, consistent with their observed increases in FLC resistance. The other catalytic site residue whose variation significantly affects FLC resistance levels (S405) lies just beyond the set predicted to be within 4.5 Å of FLC. Additionally, the variations in the Y132F and S405F single mutants have the potential to alter hydrogen bonding and/or the dimensions of the catalytic site. The remaining catalytic site residue whose variation significantly affects FLC resistance levels (I483) occurs in another part of the catalytic site where the conservative nature of its variation to valine would not be expected to alter FLC binding. In contrast, K143, whose variation to arginine induces maximum resistance to FLC, lies in a region below the heme expected to participate in electron transfer from P450 reductase.

DISCUSSION

The cytochrome P450 (CYP) superfamily of enzymes contains >2,500 members that can be roughly placed into two groups stratified by their function (25). The members of the first group metabolize a wide variety of xenobiotics, while those in the second group generally participate in key biosynthetic processes, such as sterol biosynthesis. The substrate specificity for this second group of P450s is narrow. Sterol 14α-demethylase (Cyp51 or Erg11) is considered to be the most ancient of CYP family enzymes because it is the only P450 class that is found in different kingdoms, such as animals, plants, and bacteria (26, 27). The first virally encoded CYP450 gene identified in 2009 was shown to have low-level sequence identity with previously characterized *CYP51* genes, but its function remains unknown (28). Despite the limited sequence identity (22 to 33%) shared among the Cyp51 proteins in different kingdoms (27), there are many structural similarities in the available Cyp51 crystal structures. The differences among these structures are most notable in *C. albicans* and other fungal Cyp51 proteins that contain an additional external loop between residues 428 and 459.

Recently, the Clinical Laboratory and Standards Institute (CLSI) redefined its standards for *in vitro* antifungal susceptibility testing. The current standards now consider species-specific clinical breakpoints (CBP) using established epidemiological cutoff values (ECV), defined as the upper cutoff value for wild-type MIC, pharmacokinetic (PK)-pharmacodynamic (PD) parameters, and the relationship between MIC and clinical outcome (29, 30). The current interpretation of *in vitro* susceptibility testing of *C. albicans* to fluconazole is susceptible with an MIC of ≤2 μg/ml, susceptible dose dependent with an MIC of 4 μg/ml, and resistant with an MIC of ≥8 μg/ml. The clinical breakpoints to voriconazole are defined as susceptible with an MIC of ≤0.12 μg/ml, susceptible dose dependent with an MIC of 0.25 to 0.5 μg/ml, and resistant with an MIC of ≥1 μg/ml.

In agreement with previous studies (13), the majority (87%) of our fluconazole-resistant clinical isolates carried point mutations in *ERG11* that led to at least one amino acid substitution. The substitutions in our collection were recovered in 26 distinct positions, with 21 located in previously defined hot spot regions of *CaERG11* mutations (Fig. 1) (12). Our homology model for the Erg11 protein demonstrates that these variations occur in the predicted catalytic site and extended fungus-specific external loop, as well as on the proximal surface and between the proximal surface and the heme.

In our constructed strains, most *ERG11* alleles containing single-nucleotide changes resulted in meaningful changes in the fluconazole MIC but not in the itraconazole or voriconazole MIC (Fig. 2). Among the single amino substitutions, K143R, which demonstrated the strongest increase in fluconazole MIC in this study and is predicted to occur between the proximal surface and the heme, likely affects catalytic efficiency toward the lanosterol substrate of Erg11. Y132F, which demonstrated a strong increase in fluconazole MIC and is predicted to occur in the catalytic site, likely affects fluconazole binding. Interestingly, the presence of both in the Y132F+K143R double mutant yielded the strongest increase in fluconazole MIC of any allele tested. Notably, the K143 and Y123 positions are conserved among fungal species (Fig. 1).

Among the remaining single amino acid substitutions associ-

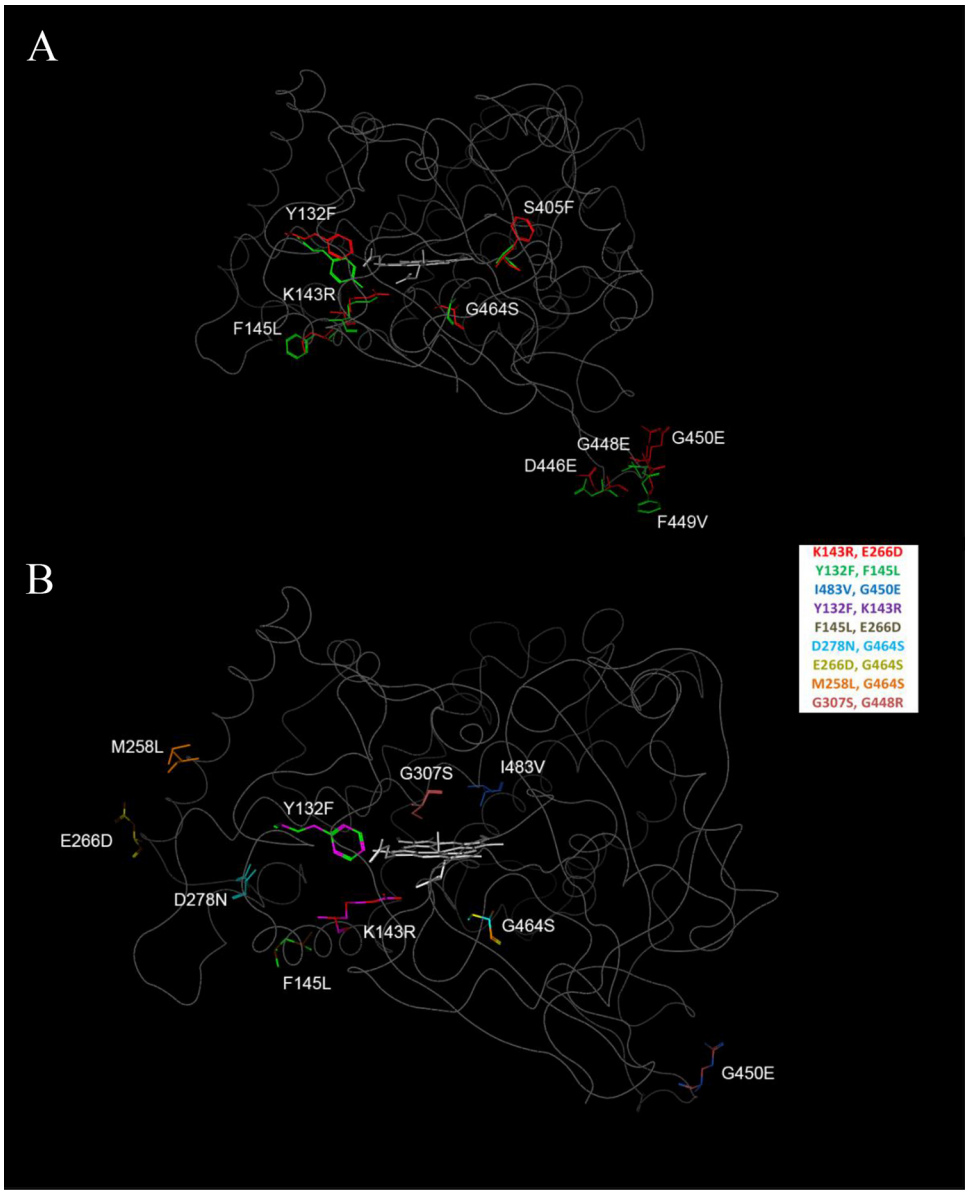

FIG 3 Mapping of mutant positions on the predicted structure for wild-type Cyp51. (A) The sequence variations in the single mutants characterized in this study are shown, with wild-type residues shown in green stick format, variant residues shown in red stick format, and heme shown in gray stick format. (B) The co-occurring sequence variations in the double mutants are shown with various colors, as identified in the legend box.

ated with fluconazole resistance, the S405F and G464S substitutions have been demonstrated to increase azole resistance when heterologously expressed in *S. cerevisiae* (14). In our collection, the S405F substitution, which was recovered in four distinct clinical isolates and as a single polymorphism only, is predicted to occur in the catalytic site and likely influences the binding of fluconazole. As documented by Kelly et al. (11) and as suggested by its position below the heme, the G464S substitution changes the heme environment and reduces its affinity for fluconazole without affecting the catalytic activity of the enzyme.

Several other single and double amino acid substitutions recovered in our collection occur in the fungus-specific external insertion loop. Sequence comparisons of this loop in different fungal species have indicated that the N-terminal portion of the loop is variable in sequence and length, while the C-terminal portion contains acidic residues followed by a more invariable portion with the motif DYG[FY]Gx[VI][ST]KG (31) corresponding to D_{446}YGFGKVSKG_{455} in the CaCyp51 protein. All four amino acid substitutions recovered in this sequence, D446E, G448E, F449V, and G450E, occur within the invariable portion.

We also examined the collective effects of multiple mutations in *ERG11* that were derived from clinical isolates. The K143R+Y132F combination resulted in the strongest increase in MIC by substantially increasing fluconazole and voriconazole MICs. This combination occurred independently in two isolates that were highly resistant to fluconazole (MIC, ≥256 μg/ml) and was accompanied by increased expression of ABC transporters *CDR1* and *CDR2* in both isolates. F145L and Y132F were the only

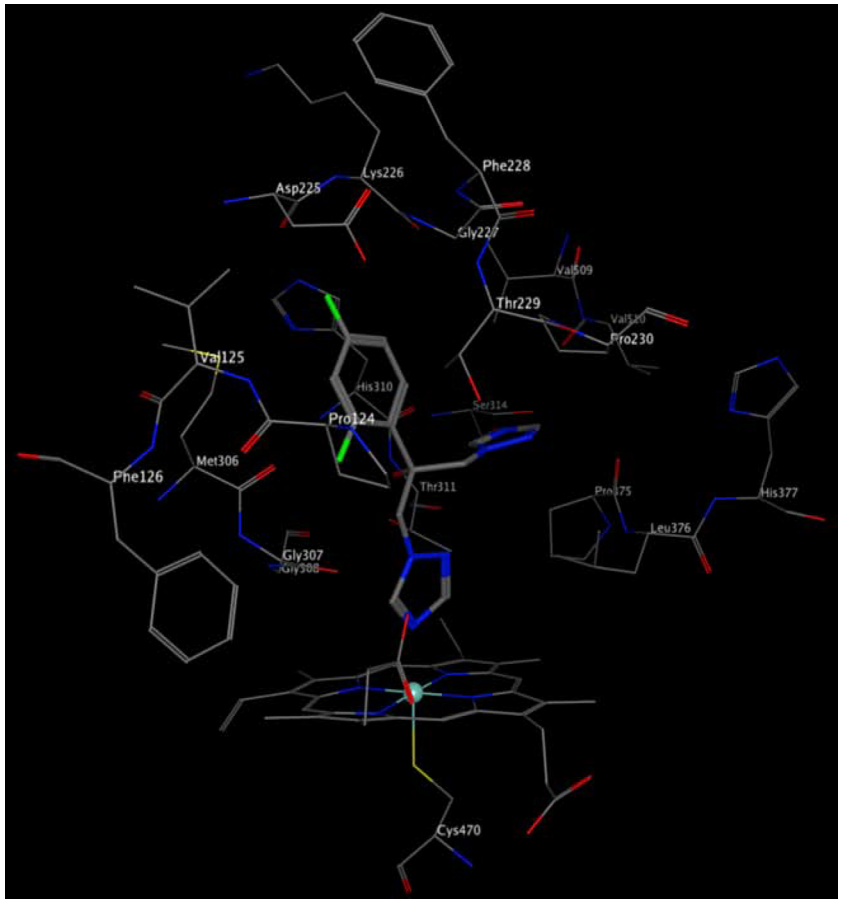

FIG 4 Predicted docking modes of fluconazole in wild-type Cyp51. FLC docked in the Cyp51 site is shown in elemental stick format, with residues within 4.5 Å shown in elemental line format.

amino acid substitutions that significantly affected MICs to all azoles. Previous modeling in *Aspergillus fumigatus* defined F145 as a position that interacts with posaconazole (32). Structurally, itraconazole is similar to posaconazole, and notably, this was the only combination of amino acid substitutions that significantly affected susceptibility to itraconazole. This combination of substitutions was also shown to catalytically impair Cyp51 when derived from a clinical *C. albicans* isolate. An investigation of Cyp51 function in *A. fumigatus* showed that positions G464S and G307 disturb the heme environment (32). This observation is in accordance with our prediction of azole binding, which indicates that G307 is located within interaction distance of FLC.

Other amino acid substitutions corresponding to I483V, M258L, and D278N occur only in combination with one of the previously mentioned single amino acid substitutions, and in all cases, they significantly increase fluconazole MICs. The I483V substitution that has not been described is predicted to be located in the catalytic site and, when combined with the G450E substitution, it increases the fluconazole MIC an additional 2-fold over that observed for the G450E substitution alone. Similar trends are observed for the M258L and D278N substitutions that in combination with the G464S substitution increase fluconazole MICs by 2-fold and 4-fold, respectively, over that observed for the G464S substitution alone. Notably, the D278E substitution previously shown to occur in azole-resistant isolates (33) is a more conserved substitution than the D278N substitution observed in our collection.

In this study, we sequenced only *ERG11* alleles from isolates exhibiting decreased susceptibilities to fluconazole; it is likely that some mutations in *ERG11* occurring in both azole-susceptible and -resistant isolates do not affect azole resistance. Another possibility is that mutations may arise in conjunction with mutations creating azole resistance that also affect fitness. Previous work has shown that specific *ERG11* mutations affect the fitness and catalytic activity of the enzyme (5, 20, 24). Further investigation of how *ERG11* mutations affect fitness is warranted.

Last, one heterozygous fluconazole-resistant isolate contained a Q21L amino acid substitution in one *ERG11* mutant allele and a wild-type *ERG11* allele. Because this substitution is located in the N-terminal transmembrane helix in membrane-bound lanosterol demethylases, anchoring this enzyme to the endoplasmic reticulum (34), its role in fluconazole resistance cannot be predicted from structural models. Our finding that the Q21L substitution has little effect on azole resistance levels when expressed in the azole-susceptible SC5314s strain indicates that mutations occurring in the transmembrane region of the Erg11 protein are unlikely to interfere with enzyme-substrate interactions.

Clearly, the susceptibility of clinical isolates is a product of the interplay of multiple mechanisms of resistance. Mutations in the *ERG11* gene have been shown to be a significant and prevalent

mechanism of resistance in *C. albicans*. Notably, in addition to carrying mutations in *ERG11*, 20 of the clinical isolates in this study also carried activating mutations in *UPC2*, which have been shown to have a combinatorial effect on azole susceptibility (24). Our data demonstrate that many *ERG11* mutations result in fluconazole resistance, but most are not as significant when tested against voriconazole or itraconazole. Susceptibility to itraconazole in particular seems to be less affected by *ERG11* mutations that produce significant resistance to fluconazole. Despite this general observation, we have identified a specific combination of amino acid substitutions that significantly reduces itraconazole and voriconazole susceptibilities.

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