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MECHANISMS OF RESISTANCE

Impact of Erg11 Amino Acid Substitutions Identified in *Candida auris* Clade III Isolates on Triazole Drug Susceptibility

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ABSTRACT *ERG11* sequencing of 28 *Candida auris* clade III isolates revealed the presence of concomitant V125A and F126L substitutions. Heterologous expression of Erg11-V125A/F126L in *Saccharomyces cerevisiae* led to reduced fluconazole and voriconazole susceptibilities. Generation of single substitution gene variants through site-directed mutagenesis uncovered that F126L primarily contributes to the elevated triazole MICs. A similar yet diminished pattern of reduced susceptibility was observed with the long-tailed triazoles posaconazole and itraconazole for the V125A/F126L, F126L, Y132F, and K143R alleles.

KEYWORDS *Candida auris*, ERG11, azole resistance, fluconazole resistance, clade III, African clade, heterologous expression, mutagenesis, short- and long-tailed triazoles

Candida auris is an emerging fungal pathogen that has spread across the globe and caused multiple health care center outbreaks. Strains of *C. auris* are divided into five genetically distinct, geographic clades: South Asian (I), East Asian (II), African (III), South American (IV), and Iranian (V) (1). Initial spread of *C. auris* to the U.S. and other parts of the world is predicted to have occurred through multiple travel-related introductions (2). Recently, several reports have shown high rates of *C. auris* candidemia in hospitalized patients with severe COVID-19 (SARS-CoV-2 infection), particularly in severely ill patients in the intensive care unit (ICU) setting (3–5). Interestingly, the pathogenicity of *C. auris* differs from other species in that it can colonize the skin, persist on hospital surfaces and on medical equipment, and transfer from person to person (6, 7). In addition, *C. auris* exhibits elevated rates of antifungal resistance. Clinical isolates that demonstrate reduced susceptibility to one or more classes of antifungals, including triazoles, polyenes (amphotericin B), and echinocandins, have been reported with triazole resistance being the most prevalent (8–10).

Triazole antifungals, such as fluconazole, voriconazole, itraconazole, and posaconazole, target the biosynthesis of fungal ergosterol specifically through inhibition of lanosterol 14-alpha-demethylase (Erg11p) that is encoded by the *ERG11* gene in yeast. Early reports identified single Erg11 substitutions (F126L, Y132F, or K143R) in strains from multiple clades (9, 11–13). These substitutions were highlighted due to their connection to triazole resistance within other species of *Candida*, specifically *C. albicans*

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TABLE 1 Triazole drug susceptibility and Erg11 profiles of 28 *C. auris* clade III clinical isolates

Origin	Isolate[a]	Specimen	Erg11	MIC[b] (μg/ml)			
				FLC	VRC	POS	ITC
AR bank	AR-0381	N/A	WT	4	0.03	<0.03	0.03
	AR-0382	N/A	WT	4	0.03	0.06	0.06
	AR-0383	N/A	V125A/F126L	>128	1	0.03	0.125
	AR-0384	N/A	V125A/F126L	>128	1	0.06	0.25
South Africa	SA1	CVC tip	V125A/F126L	>128	1	0.125	0.25
	SA2	Blood	V125A/F126L	>128	0.5	0.125	0.5
	SA3	CVC tip	V125A/F126L	>128	1	0.03	0.5
	SA4	Blood	V125A/F126L	>128	1	0.03	0.5
	SA5	Tracheal aspirate	V125A/F126L	>128	2	0.03	0.5
	SA6	Blood	V125A/F126L	>128	1	0.03	0.5
	SA7	Urine	V125A/F126L	>128	1	0.125	0.5
	SA8	Urine	V125A/F126L	>128	1	0.03	0.25
	SA9	Urine	V125A/F126L	>128	1	0.03	0.5
	SA10	Blood	V125A/F126L	>128	1	0.125	0.5
	SA11	Blood	V125A/F126L	>128	1	0.03	1
	SA12	Urine	V125A/F126L	>128	1	0.03	0.25
	SA13	Urine	V125A/F126L	8	1	0.03	0.25
	SA14	Urine	V125A/F126L	>128	2	0.03	0.5
	SA15	Tracheal aspirate	V125A/F126L	8	1	0.03	0.5
	SA16	Tracheal aspirate	V125A/F126L	8	1	0.03	0.5
	SA17	Urine	V125A/F126L	>128	1	0.03	0.25
	SA18	Urine	V125A/F126L	128	0.5	0.03	0.5
	SA19	Urine	V125A/F126L	128	2	0.03	0.25
	SA22	Urine	V125A/F126L	128	0.5	0.25	0.5
	SA23	Urine	V125A/F126L	>128	8	0.25	0.5
Australia	A3	Sternum	V125A/F126L	>128	1	0.25	1
	A4	Sternum	V125A/F126L	>128	1	0.25	1
	A6	Axilla & groin	V125A/F126L	>128	0.25	0.125	1
	A7	Axilla & groin	V125A/F126L	>128	0.25	0.25	1
	A8	Catheter specimen of urine (CSU)	V125A/F126L	>128	0.5	0.25	1

[a]For reference, susceptibility results are presented for two Erg11 wild-type (WT) strains: AR-0381 (clade II) and AR-0382 (clade I).
[b]FLC, fluconazole; VRC, voriconazole; POS, posaconazole; ITC, itraconazole.

(14). In our previous study (15), we identified and analyzed *C. auris ERG11* mutations in clinical isolates of clades I and IV. Using a heterologous expression system, we directly linked the Y132F and K143R Erg11 substitutions to fluconazole and voriconazole resistance, whereas other alterations, including I466M, Y501H, and clade-specific polymorphisms, were not associated with elevated MICs.

Here, we investigated triazole resistance in 28 clinical isolates of *C. auris* clade III obtained from South Africa ($n = 21$), Australia ($n = 5$), and the CDC and FDA Antimicrobial Resistance (AR) Isolate Bank ($n = 2$). *ERG11* was amplified and sequenced as described before (15). In agreement with recent reports (8, 16, 17), we identified two *ERG11* mutations, T374C and T376C, that lead to two amino acid substitutions, V125A and F126L, respectively, in all 28 isolates (Table 1). Antifungal susceptibility testing was performed in triplicate for each clinical isolate according to CLSI methodology (18, 19) with *C. parapsilosis* (ATCC 22019) and *C. krusei* (ATCC 6258) used as quality control strains. MICs were interpreted using tentative breakpoints as suggested by the CDC (https://www.cdc.gov/fungal/candida-auris/c-auris-antifungal.html). These isolates demonstrated reduced triazole susceptibilities, specifically to fluconazole and voriconazole (Table 1). Of note, clinical isolates SA13, SA15, and SA16 demonstrated fluconazole MICs in the susceptible range of less than 32 μg/ml, despite containing the same *ERG11* mutations as the other strains (Table 1). This may point to other mechanisms of differential triazole resistance and/or additional alterations in these isolates that specifically influence the fluconazole-Erg11p interaction. Further analyses on these strains are under way.

Using the same approach as in reference (15), we cloned the Erg11 allele (V125A/F126L) from isolate AR-0384 onto pRS416, a low-copy-number plasmid that contains

TABLE 2 Triazole susceptibilities of *S. cerevisiae* strains that express *C. auris* Erg11 plasmid constructs

	MIC[a] (μg/ml)			
Erg11 allele	**FLC**	**VRC**	**POS**	**ITC**
Empty vector	16	0.12	0.25	0.5
Wild type (clade I)	16	0.25	0.25	1
Wild type (clade IV)	16	0.12	0.25	1
I466M	16	0.25	0.5	2
Y132F	128	2	0.5	4
K143R	64	1	0.5	2
V125A/F126L	64	1	0.5	2
Wild type (clade III)	16	0.25	0.25	1
V125A	16	0.25	0.25	1
F126L	64	1	0.5	4

[a]MICs were obtained in both nutrient-rich YPD (yeast extract, peptone, dextrose) and nutrient-limited SD-Ura broth media. We observed a 2-fold or less difference between these media. Fluconazole and voriconazole MICs of the first six strains were previously reported (15); however, these MICs were repeated in tandem with the newly engineered strains and are presented here for comparison. FLC, fluconazole; VRC, voriconazole; POS, posaconazole; ITC, itraconazole.

the *S. cerevisiae URA3* marker (ATCC 87521). This construct was then expressed in a haploid strain of *S. cerevisiae* (BY4741; ATCC 201388) that is auxotrophic for uracil biosynthesis. This heterologous system allowed us to focus solely on the effects of *ERG11* mutations on triazole susceptibilities. Multiple clones were passaged on selective medium (synthetic defined medium lacking uracil; SD-Ura), screened by PCR, and the resulting plasmid sequences verified (for primers, see reference [15]). *S. cerevisiae* that expressed *C. auris* Erg11-V125A/F126L demonstrated elevated MICs to fluconazole (64 μg/ml) and voriconazole (1 μg/ml). In comparison, expression of an empty vector or Erg11 wild-type alleles from other clades yielded MICs 4- to 8-fold more susceptible ($\leq$16 μg/ml to fluconazole; $\leq$0.25 μg/ml to voriconazole) (Table 2).

To further dissect the specific role of V125A and F126L substitutions in triazole resistance, we designed mutagenic primers to individually revert each amino acid substitution (Fig. 1). A Phusion site-directed mutagenesis kit (Thermo Scientific; cat. no. F541) was used to introduce the desired wild-type mutations. The resulting *C. auris* Erg11-V125A and Erg11-F126L plasmid constructs were expressed in *S. cerevisiae*. In addition, we performed two consecutive rounds of site-directed mutagenesis to produce a strain that carried neither substitution (Erg11-V125/F126) (Fig. 1D and E). This strain represented a *de facto* clade III wild-type allele. Plasmid sequences of all alleles were confirmed. Subsequent triazole susceptibility assays revealed that cells expressing F126L alone exhibited elevated MICs, similar to V125A/F126L, while V125A alone led to MICs similar to that of the wild-type alleles (Table 2). Our engineered, clade III wild-type allele yielded susceptible MICs, allowing us to conclude that the *ERG11* mutations, as opposed to expression levels, were mainly contributing to the observed decreased in susceptibility.

Drug binding and cloning studies have demonstrated that certain *ERG11* mutations in *S. cerevisiae* and *C. albicans* influence susceptibility to all triazoles, while other mutations lead to decreased susceptibility to only short- or long-tailed triazoles (20–22). Therefore, in addition to fluconazole and voriconazole (short-tailed triazoles), we tested each of our strains to determine susceptibility to posaconazole and itraconazole (long-tailed triazoles) (Table 2). Changes in the posaconazole and itraconazole MICs were minimal, although consistent, and with 2- to 4-fold differences between the "resistant" alleles (V125A/F126L, Y132F, or K143R) and the wild-type alleles (Table 2). These results are in alignment with the minimal differences observed in clinical isolates (Table 1) and to those of previous studies that analyzed Y132F and K143R or equivalent changes in *C. albicans* and *S. cerevisiae* (21, 23).

Crystallization of *C. albicans Erg11* identified residue 126, and the equivalent residue in *S. cerevisiae*, as being located within the enzyme's active site and a likely player in substrate binding (24, 25). Furthermore, the authors from that study predicted that

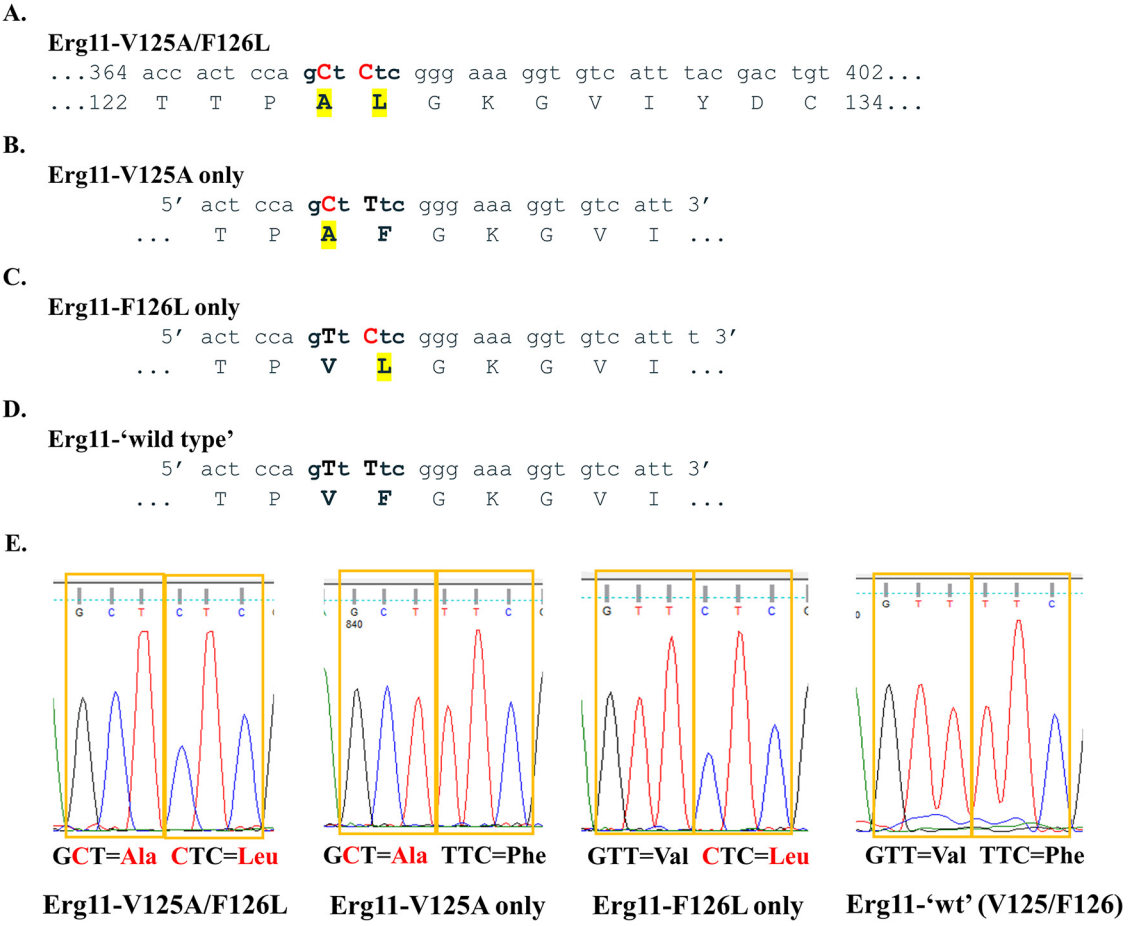

FIG 1 Molecular dissection of *C. auris* Erg11 V125A and F126L amino acid substitutions. (A) Region of *C. auris* clade III *ERG11* DNA that displays nucleotide mutations (T374C/T376C) in red and resulting protein alterations (V125A/F126L) in yellow highlight. (B) Forward mutagenic primer used to revert leucine (L) back to phenylalanine (F). After mutagenesis, this construct contained only V125A (pCauErg11-V125A). (C) Forward mutagenic primer used to revert alanine (A) back to wild-type valine (V). After mutagenesis, this construct contained only F126L (pCauErg11-F126L). (D) Forward mutagenic primer used to revert leucine (L) back to phenylalanine (F) using the pCauErg11-F126L plasmid as a template. After mutagenesis, this construct contained both wild-type nucleotides and amino acids (pCauErg11-'wt'). (E) Plasmid sequencing chromatograms of relevant codons corresponding to the 125th and 126th amino acids following mutagenesis and propagation in *Escherichia coli*.

alteration of this residue would likely reduce affinity for all triazole drugs but would do so most extensively for short-tailed azoles (24). Because *C. auris* clade III isolates described in the literature contain both V125A and F126L substitutions, it is likely that these two mutations occurred at nearly the same time in the evolution of this clade. The V125A substitution may simply be a passenger mutation. Alternatively, V125A may increase the stability of the Erg11 enzyme or be advantageous for the yeast in another way and/or in combination with other alterations (e.g., *ERG11* copy number variants [8]). Studies have since identified *TAC1b* transcription factor mutations, linked to increased expression of drug efflux pumps (e.g., *CDR1* and/or other unidentified transporters), as an alternate mechanism of triazole resistance in *C. auris* (26–28). Additionally, a recent report demonstrated an additive effect that concomitant *ERG11* (F444L) and *TAC1b* mutations can have on triazole resistance (29). Of note, the SA23 isolate demonstrated unusually high triazole MICs, which were most noticeable for voriconazole. It is probable that additional mechanisms of triazole resistance are involved and are being investigated in an ongoing study.

In conclusion, the *ERG11* allele found in *C. auris* clade III isolates directly contributes to reduced triazole susceptibility, in particular to fluconazole and voriconazole. Moreover, our mutagenic experiments revealed that the F126L substitution was primarily responsible for

the elevated triazole MICs. Results of this study further improve our understanding of triazole resistance mechanisms in *C. auris*, which can have a direct impact on diagnostic and treatment practices.

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We declare no conflicts of interest.

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