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Precise genome editing underlines the distinct contributions of mutations in *ERG11*, *ERG3*, *MRR1*, and *TAC1* genes to antifungal resistance in *Candida parapsilosis*

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ABSTRACT *Candida parapsilosis* has recently emerged as a major threat due to the worldwide emergence of fluconazole-resistant strains causing clonal outbreaks in hospitals and poses a therapeutic challenge due to the limited antifungal armamentarium. Here, we used precise genome editing using CRISPR-Cas9 to gain further insights into the contribution of mutations in *ERG11*, *ERG3*, *MRR1*, and *TAC1* genes and the influence of allelic dosage to antifungal resistance in *C. parapsilosis*. Seven of the most common amino acid substitutions previously reported in fluconazole-resistant clinical isolates (including Y132F in *ERG11*) were engineered in two fluconazole-susceptible *C. parapsilosis* lineages (ATCC 22019 and STZ5). Each mutant was then challenged *in vitro* against a large array of antifungals, with a focus on azoles. Any possible change in virulence was also assessed in a *Galleria mellonella* model. We successfully generated a total of 19 different mutants, using CRISPR-Cas9. Except for R398I (*ERG11*), all remaining amino acid substitutions conferred reduced susceptibility to fluconazole. However, the impact on fluconazole *in vitro* susceptibility varied greatly according to the engineered mutation, the stronger impact being noted for G583R acting as a gain-of-function mutation in *MRR1*. Cross-resistance with newer azoles, non-medical azoles, but also non-azole antifungals such as flucytosine, was occasionally noted. Posaconazole and isavuconazole remained the most active *in vitro*. Except for G583R, no fitness cost was associated with the acquisition of fluconazole resistance. We highlight the distinct contributions of amino acid substitutions in *ERG11*, *ERG3*, *MRR1*, and *TAC1* genes to antifungal resistance in *C. parapsilosis*.

KEYWORDS *Candida parapsilosis*, antifungal resistance, amino acid substitutions, fluconazole, agricultural azoles, precise genome editing, CRISPR-Cas9, *Galleria mellonella*, *ERG11*, *ERG3*, *MRR1*, *TAC1*, resistome

Considering the rise of fungal diseases, worrisome trends in antifungal resistance, and unmet needs, the WHO recently published the first-ever fungal priority pathogens list (1). *Candida parapsilosis* which is one of these priority pathogens is a diploid ascomycetous yeast and a natural commensal of the human skin (2, 3). Under certain circumstances or in patient populations including those from Intensive Care Units (ICU), having hematological malignancies, or preterm neonates, this opportunistic species can also act as a pathogen causing invasive candidiasis. *C. parapsilosis* is among the top four most prevalent species causing candidemia according to national/international surveillance networks (4, 5). In a few years, *C. parapsilosis* has emerged as a serious concern in many countries due to a dramatic increase in fluconazole resistance along with its ability to cause clonal outbreaks in hospitals which can be explained by its potential for interhuman transmission (6). This situation is of concern as this species is,

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per se, less susceptible to echinocandins than other yeast species and further reinforced by the recent description of micafungin-resistant and multidrug-resistant (MDR) strains (7, 8). Due to the limited antifungal armamentarium, managing the patients infected by MDR strains can be challenging, leaving amphotericin B as the last resort (6).

As opposed to *Candida albicans* and *Nakaseomyces glabratus* (i.e., *Candida glabrata*), the molecular mechanisms leading to antifungal resistance in *C. parapsilosis* are only beginning to be understood. As in other *Candida* species, amino acid substitutions in the *ERG11* gene, encoding lanosterol demethylase (a major enzyme of the ergosterol biosynthesis pathway and the cellular target of azoles), are frequently observed in fluconazole-resistant clinical isolates, as recently reviewed (9). Y132F in *ERG11* is by far the most common amino acid substitution reported (10–18). In addition, active efflux due to the overexpression of two distinct efflux pumps, namely Mdr1 (Major Facilitator superfamily) and Cdr1 (ATP-binding cassette superfamily), also seems to play a pivotal role. Actually, overexpression of *MDR1/CDR1* genes is driven by gain of function mutations in *TAC1* and *MRR1*, respectively, two zinc-finger transcription factors, with new mutations being regularly described (19, 20). Mutations in *ERG3*, a gene involved in the ergosterol biosynthesis pathway, have been reported and unexpectedly associated with reduced susceptibility to echinocandins (21). Although closely related to *C. albicans* within the CTG clade in the Saccharomycotina, a recent study supports a unique genetic regulation network in *C. parapsilosis* with *MRR1* governing both *CDR1* and *MDR1* (22). Finally, many questions related to its antifungal resistance are still unresolved, such as: (i) the real contribution of mutations identified in fluconazole-resistant clinical isolates to the last generation of azoles and the risk of cross resistance between azoles and non-azole antifungals; (ii) the impact of allelic dosage and genetic make-up of the clinical strains, on the antifungal susceptibility profiles; (iii) the potential fitness costs associated with the acquisition of azole resistance. Providing answers to these key questions would be a game-changer allowing to increase our understanding of antifungal resistance and hopefully improve patient care. To address these burning questions, we took advantage of precise genome editing using CRISPR-Cas9 to delineate the direct contribution of mutations in *ERG11*, *ERG3*, *MRR1*, and *TAC1* genes, all previously reported from clinical isolates, to antifungal resistance in *C. parapsilosis*.

MATERIALS AND METHODS

Clinical isolates

Two fluconazole-susceptible *C. parapsilosis* genetic background strains were used in this study, the ATCC 22019 reference strain and a clinical strain (STZ5). Both strains (referred below as the "parental") and their respective CRISPR mutants engineered in this study (Table 1) were cryopreserved at −80°C (15% glycerol final concentration).

Precise genome editing in *C. parapsilosis* using CRISPR-Cas9

Seven of the most prevalent single nucleotide polymorphisms (SNPs) causing amino acid substitutions described in fluconazole-resistant *C. parapsilosis* clinical isolates (23) were selected: A395T (Y132F), A428G (K143R), and G1193T (R398I) in *ERG11*, G1949A (G650E) and T2933G (L978W) in *TAC1*, G1747A (G583R) in *MRR1*, and G331A (G111R) in *ERG3*. To evaluate their individual contribution to antifungal resistance as well as the influence of allelic dosage, we aimed to engineer heterozygous (mutation in a single allele) and homozygous mutants (mutation in both alleles) in each of the two parental strains (ATCC 22019 and STZ5). Except for *ERG11* (GQ302972.1), nucleotide sequences of *MRR1*, *TAC1*, and *ERG3* (CPAR2_807270, CPAR2_303510 and CPAR2_105550, respectively) required for primers design were retrieved from the Candida Genome Database (http://www.candidagenome.org/).

A CRISPR-Cas9 markerless genome editing technique, specifically developed for *C. parapsilosis* and relying on an episomal and non-integrative plasmid (pCP-tRNA), was

TABLE 1 *Candida parapsilosis* strains used in this study

Strain	Genotype	Origin
ATCC 22019	Wild-type	Reference strain
STZ5	Wild-type	Clinical strain
CP-A5/D3 (ATCC 22019 background)	*ERG11*$^{Y132F/WT}$	This study
CP-A5/F3 (ATCC 22019 background)	*ERG11*$^{Y132F/Y132F}$	This study
CP-A5/A4 (ATCC 22019 background)	*ERG11*$^{K143R/WT}$	This study
CP-A5/H3 (ATCC 22019 background)	*ERG11*$^{K143R/K143R}$	This study
CP-A5/F4 (ATCC 22019 background)	*ERG11*$^{R398I/R398I}$	This study
CP-A5/C5 (ATCC 22019 background)	*TAC1*$^{G650E/G650E}$	This study
CP-A5/D5 (ATCC 22019 background)	*TAC1*$^{L978W/L978W}$	This study
CP-A5/I5 (ATCC 22019 background)	*MRR1*$^{G583R/WT}$	This study
CP-A5/J6 (ATCC 22019 background)	*MRR1*$^{G583R/G583R}$	This study
CP-A5/A5 (ATCC 22019 background)	*ERG3*$^{G111R/G111R}$	This study
CP-A5/B4 (STZ5 background)	*ERG11*$^{Y132F/WT}$	This study
CP-A5/C4 (STZ5 background)	*ERG11*$^{Y132F/Y132F}$	This study
CP-A5/E4 (STZ5 background)	*ERG11*$^{K143R/WT}$	This study
CP-A5/D4 (STZ5 background)	*ERG11*$^{K143R/K143R}$	This study
CP-A5/G4 (STZ5 background)	*ERG11*$^{R398I/R398I}$	This study
CP-A5/B7 (STZ5 background)	*TAC1*$^{G650E/G650E}$	This study
CP-A5/I7 (STZ5 background)	*TAC1*$^{L978W/L978W}$	This study
CP-A5/J4 (STZ5 background)	*MRR1*$^{G583R/G583R}$	This study
CP-A5/B5 (STZ5 background)	*ERG3*$^{G111R/G111R}$	This study

used to construct each mutant strain (24). Twenty base pair RNA guides allowing to engineering each SNP were first designed using EuPaGDT (http://grna.ctegd.uga.edu/). Briefly, each guide was cloned into the SapI-digested pCP-tRNA plasmid. Plasmids were then propagated in *Escherichia coli* (DH5α strain), and selection was performed on LB with Ampicillin (100 µg/mL final concentration). Plasmids were stored at −20°C before transformation in *C. parapsilosis*. Hundred base pair double-strand DNA repair templates (i.e., 50 bp of homology arms) were designed to engineer the desired mutations by homologous recombination. Five microgram of pCP-tRNA plasmid containing the appropriate guide and 25 µL of repair template were then used for the chemical transformation of each *C. parapsilosis* genetic background, as previously described (24). The mutant selection was performed on YPD agar supplemented with nourseothricin (200 µg/mL final concentration) after 3 days at 30°C. Plasmid removal was obtained by subculturing the transformants onto the YPD medium without nourseothricin. The presence of the mutation of interest was verified by Sanger sequencing using primers designed to amplify 200 bp upstream and downstream of the desired mutation. All primers designed to produce the guides, repair templates, or used for Sanger sequencing are listed in Table S1 and were produced by Eurofins Genomics (Ebersberg, Germany).

In vitro antifungal susceptibility to systemic antifungals

Fluconazole susceptibility of each CRISPR mutant was first determined by the EUCAST reference microdilution method (EUCAST E.DEF 7.3.2 April 2020). Fluconazole powder was purchased from Carbosynth (Compton, UK). Plates were incubated at 35°C for 24 h, and fungal growth was evaluated by measuring the absorbance at 530 nm on a SpectroStar nano spectrophotometer (BMG Labtech, Ortenberg, Germany). Minimal Inhibitory Concentrations (MICs) were determined as the lowest drug concentrations yielding a ≥50% growth inhibition relative to the growth of the drug-free control well. In addition to the *C. parapsilosis* ATCC 22019 which also acted as a control strain, the *Candida krusei* ATCC 6258 was included in each set of experiments. MICs were then categorized according to the EUCAST guidelines (S ≤ 2 µg/mL; R > 4 µg/mL). In addition, MICs of the mutants and their parental strains (e.g., ATCC 22019 and STZ5)

were also assessed for four triazole antifungals (fluconazole, voriconazole, posaconazole, and isavuconazole), micafungin, amphotericin B, and flucytosine using a gradient strip technique (Etest bioMérieux, France, except for isavuconazole, Liofilchem, Italy). MICs were read after 48 h incubation at 35°C, using an 80% inhibition endpoint for azoles and micafungin, a 90% endpoint for flucytosine, and full inhibition for amphotericin B. MICs were then categorized as susceptible (S), SDD-intermediate (SDD or I), or resistant (R) using the CLSI breakpoints for fluconazole (S ≤ 2 µg/mL; SDD 3–6 µg/mL; R ≥ 8 µg/mL) and voriconazole (S ≤ 0.125 µg/mL; I 0.19–0.75 µg/mL; R ≥ 1 µg/mL), according to the manufacturer's instructions. Finally, isolates with MICs above amphotericin B and posaconazole epidemiological cut-off values (1 and 0.25 µg/mL, respectively) were considered non-wild type.

In vitro susceptibility to topical antifungals

In vitro susceptibility to three imidazole antifungals, econazole (EC, 50 µg), clotrimazole (CTR, 50 µg), and miconazole (MCZ, 50 µg) was determined by a disk diffusion technique on Casitone agar plates, according to the manufacturer's instructions (Bio-Rad, Marnes-la-Coquette, France). Plates were incubated at 32°C for 24 h. Diameters were measured at zone edges corresponding to a partial inhibition and followed by categorization performed according to the manufacturer's instructions.

In vitro susceptibility to non-medical azoles

Cross resistance with agricultural azoles was also evaluated using a spot assay technique. To this aim, four dilutions of each mutant strain were spotted on YPD agar plates supplemented with either the triazole epoxiconazole or imidazoles namely prochloraz or imazalil (0.125 µg/mL, 4 µg/mL, and 0.5 µg/mL, final concentrations, respectively). Fungal growth was assessed visually after 48 h incubation at 30°C.

Sterol composition of plasma membrane

Analysis of membrane sterol composition was conducted by GC–MS as previously described (25). Briefly, fungal cells were inoculated into 25 mL of YPD 20% glucose broth (Sigma Aldrich, France) and incubated at 30°C under agitation (130 rpm) for 24 h. Cells were then collected by centrifugation at 1,500 g (room temperature) and washed twice with 5 mL of phosphate-buffered saline (PBS) 1×. The pellet was suspended in 3 mL of fresh ethanolic potassium hydroxide solution (25 g of KOH, 36 mL of distilled water, and brought to 100 mL with 100% ethanol). Then, the suspension was vortexed for 1 min, and saponification was performed at 80°C for 90 min. Sterols were then extracted by adding 3 mL of hexane, and the organic phase was washed by adding 1 mL of sterile water. The final organic phase was transferred to a new collection tube after drying with Na_2SO_4 and a 5 min centrifugation step at 2,000 rpm. An equivalent of 100 mg the fungal biomass was transferred into a glass tube, and 2.5 µg of cholesterol was added as an internal standard. Hexane was evaporated by heating at 80°C. Sterols were derivatized with 100 µL of *N*-Methyl-*N*-(trimethyl)silyltrifluoroacetamide (Sigma-Aldrich, France) during 30 min at room temperature. The solvent was then evaporated at 80°C under air flux. Dried sterols were solubilized into 200 µL dichloromethane and stored at −20°C until analysis.

Sterols as TMS derivatives were analyzed by GC-MS using an Agilent 7890A GC system, with an SLB-5 column (30 m × 0.25 mm, 0.25 µM, Sigma-Aldrich, France) coupled with a mass detector (Agilent 5975C inert MSD – E.I. 70 eV). One microliter of each sample was injected in splitless mode at 250°C. The carrier gas was helium at a flow rate of 1.2 mL/min. The oven was set at 150°C for 0.5 min and then raised to 290°C at 50°C/min and from 290°C to 305°C at 2°C/min hold for 7 min, and finally 10°C/min to 315°C and hold for 16 min. Sterols were identified via their electron ionization fragmentation pattern, compared to published data. Results are expressed as percent of total sterols area. Relative Retention Times were expressed as the ratio of the retention time of each sterol to that of cholesterol. Experiments were performed twice.

Fitness and *in vivo* virulence

The *Galleria mellonella* larvae used in the study were produced in our laboratory according to a previous study with slight modifications (26). Homogeneous groups of 10 larvae of 260–320 mg (sixth developmental stage) were used, and at least two independent experiments were conducted for each strain. Inoculum suspensions were prepared in 1× PBS from fresh cultures (24 h) grown on Sabouraud Dextrose Agar and adjusted to 5×10^8 cells/mL. Ten microliter of this adjusted inoculum was then inoculated into the penultimate proleg on the left side using a 0.3 mL 30G insulin syringe (BD Medical - USA). Larvae were incubated in the dark at 35°C. Survival, defined by the lack of movement in response to a stimulus, with or without discoloration, was determined every day and for a duration of 5 days. Larvae injected with PBS were used as the control. Survival of each mutant was then compared to its parental strain, each lineage being analyzed independently. The survival function was estimated by the Kaplan-Meier method. For each *C. parapsilosis* lineage (ATCC 22019 or STZ5), log-rank (Mantel-Cox) tests were used to compare survival functions between mutant strains. Likewise, Cox proportional hazards regression analyses were performed to study the association between each amino acid substitution and the survival of larvae; results were expressed as the corresponding log-rank *P* values, hazard ratios (HRs), and their 95% confidence intervals. Statistical significance was defined as *P*-value < 0.05. Statistical analyses and graphs were performed in R Statistical Software version 4.2.2 (R Core Team 2022), using the survminer and GGally packages.

RESULTS

All amino acid substitutions of interest were successfully engineered in both alleles for each *C. parapsilosis* genetic background. In addition, heterozygous mutants were also obtained for amino acid substitutions in the *ERG11* (Y132F and K143R) and *MRR1* (G583R). All 19 CRISPR mutants engineered for this study are listed in Table 1.

Impact on fluconazole susceptibility depends on the amino acid substitution and its allelic dosage

With the exception of R398I (*ERG11*), all mutations leading to amino acid substitutions engineered in *ERG11*, *TAC1*, and *MRR1* were individually associated with a marked decrease in fluconazole susceptibility which translated into an increased MIC (Table 2). Homozygous mutants displayed higher fluconazole MIC than their heterozygous relatives, whatever the SNP and the genetic background considered. However, mutants engineered in *C. parapsilosis* ATCC 22019 tend to display higher MICs than STZ5 mutants, reflecting the different basal fluconazole susceptibility of their parental strains. The strongest impact was noted for $MRR1^{G583R/G583R}$, which was associated with a huge increase in fluconazole MIC, in both genetic backgrounds (64 µg/mL and ≥64 µg/mL, respectively). Overall, according to the EUCAST breakpoints, most amino acid substitutions conferred reduce fluconazole susceptibility or resistance, in at least one background strain. Finally, we also engineered $ERG3^{G111R/G111R}$ which was associated with a prominent trailing growth *in vitro*, in both genetic backgrounds. Analysis of membrane sterols by GC-MS demonstrated that this substitution leads to Erg3 impairment, as evidenced by a marked decrease in membrane ergosterol (below 15% of total sterols, compared to >75% in their parental relatives) and an accumulation of intermediate sterols, mostly ergosta-7,22-dienol (±60%) and to a lesser extent ergosta-7-enol (Fig. 1).

Some substitutions can alter the susceptibility to other systemic azoles and non-azole antifungals

We then used a gradient strip method to assess if variations in fluconazole susceptibility resulting from the acquisition of the aforementioned substitutions may trigger cross resistance to other systemic azoles, namely voriconazole, posaconazole, and isavuconazole. As observed with the EUCAST method, the susceptibility of each individual

TABLE 2 *In vitro* susceptibility of the CRISPR mutants comparatively with their parental strains (ATCC 22019 or STZ5) to fluconazole, determined by the EUCAST reference method[a,b]

Amino acid substitutions	Fluconazole minimal inhibitory concentrations (µg/mL; EUCAST method)	
	Candida parapsilosis ATCC 22019	*Candida parapsilosis* STZ5
None (background strain)	2 (S)	0.5 (S)
ERG11$^{Y132F/WT}$	**8 (R)**	1 (S)
ERG11$^{Y132F/Y132F}$	**16 (R)**	**4 (I)**
ERG11$^{K143R/WT}$	**8 (R)**	2 (S)
ERG11$^{K143R/K143R}$	**16 (R)**	**4 (I)**
ERG11$^{R398I/R398I}$	2 (S)	0.5 (S)
TAC1$^{G650E/G650E}$	**8 (R)**	2 (S)
TAC1$^{L978W/L978W}$	**16 (R)**	**4 (I)**
MRR1$^{G583R/WT}$	**16 (R)**	–[c]
MRR1$^{G583R/G583R}$	**64 (R)**	**>64 (R)**
ERG3$^{G111R/G111R}$	[d]	[d]

[a]S: susceptible; R: resistant; I: Susceptible, Increase exposure.
[b]Non -susceptible MICs (i.e., above the susceptibility breakpoint, when available) are depicted in bold face.
[c]"–": mutant not available (MIC not performed).
[d]Trailing growth.

mutant to fluconazole was also dependent on the allelic dosage and basal MIC of the parental strain (Table 3). Again, *MRR1*$^{G583R/G583R}$ conferred the highest increase in fluconazole MIC as depicted in Fig. 2A, which also illustrates the stepwise development of fluconazole resistance in relation to the allelic dosage (up to >170 fold-MIC increase for the homozygous mutant). Of note, introducing Y132F or K143R in a single *ERG11* allele or substitutions in *TAC1* and *MRR1* conferred reduced susceptibility to fluconazole. However, introducing these substitutions in both alleles (i.e., homozygous) almost always led to fluconazole resistance, as depicted for Y132F in Fig. 2A. Regarding other systemic azoles, voriconazole was the most impacted, whereas posaconazole and isavuconazole *in vitro* activities seemed only moderately affected by mutations in *ERG11*, *TAC1*, and *MRR1*. *ERG11*$^{Y132F/Y132F}$ conferred reduced susceptibility or resistance to voriconazole (depending on the genetic background). In stark contrast and whatever the background, *MRR1*$^{G583R/G583R}$ conferred resistance to voriconazole and was responsible for an increase in posaconazole/isavuconazole MICs, especially in the STZ5 background. Beyond systemic azoles, we also observed that introducing *MRR1*$^{G583R/G583R}$ led to a fourfold to eightfold increase in flucytosine MIC (Fig. 2B), in both genetic backgrounds, whereas amphotericin B and micafungin susceptibility remained unchanged (data not shown). Finally, as for fluconazole, a prominent trailing growth of *ERG3*$^{G111R/G111R}$ mutants was also evidenced for the other systemic azoles (illustrated in Fig. 3 for posaconazole) in both backgrounds. This trailing growth, which was not observed with the parental strains, was only visible after 48 h of incubation. Of note, *ERG3*$^{G111R/G111R}$ also triggered a slight increase in both amphotericin B and micafungin MICs, with microcolonies observed within the ellipse of inhibition after 48 h incubation (Fig. 4).

Imidazoles globally retain *in vitro* antifungal activity on *C. parapsilosis* fluconazole-resistant strains

After systemic triazoles, we then assessed the impact of the aforementioned substitutions on the activity of three imidazoles. To this end, the *in vitro* susceptibility of eight mutants (four for each genetic background) was evaluated by disk diffusion. Compared with their parental relatives, all strains carrying substitutions in *ERG11*, *TAC1*, and *MRR1* displayed reduced susceptibility to miconazole. On the contrary, despite reduced zone diameters, all strains remained susceptible to econazole and clotrimazole (Table 4).

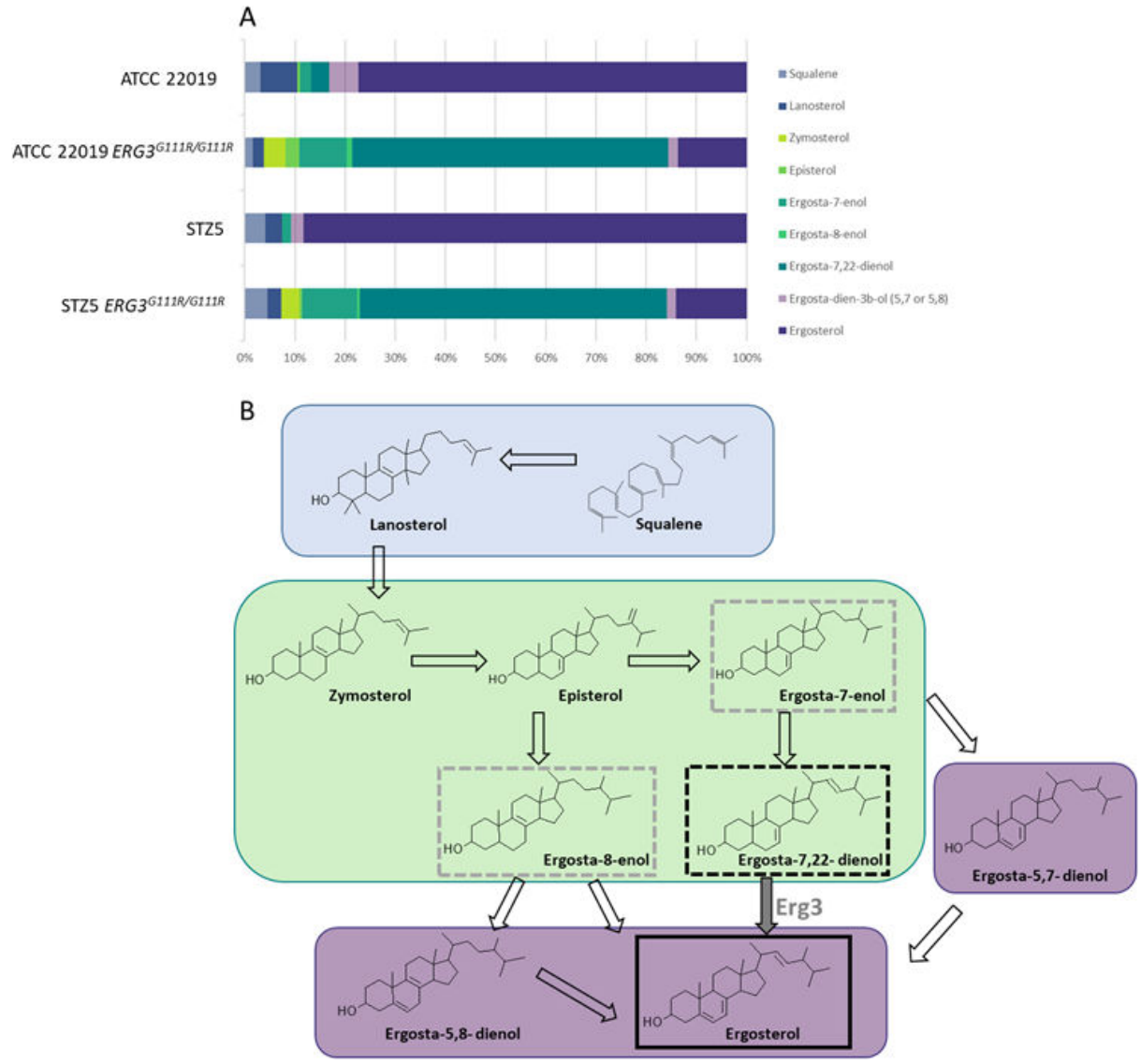

FIG 1 (A) Relative membrane sterol composition (%) of *C. parapsilosis ERG3*$^{G111R/G111R}$ strains compared with their parental relatives (ATCC 22019 and STZ5). (B) Simplified ergosterol biosynthesis pathway with sterols precursors in the blue box, sterol intermediates in the green box, and end products in the purple box. Dotted lines depict sterols accumulating in *ERG3* mutant strains.

Amino acid substitutions in *ERG11*, *TAC1*, and *MRR1* not only confer resistance to medical azoles but also increase resistance to agricultural azoles

We then determined if substitutions altering the activity of *C. parapsilosis* to medical azoles could also have an impact on the susceptibility to non-medical azoles, namely agricultural azoles widely used as fungicides. As shown in Fig. 5, compared to their parental relatives, all mutant strains displayed increased resistance to epoxiconazole and imazalil. A weaker effect was observed for the *ERG11*$^{K143R/K143R}$ mutants for epoxiconazole. Similar results were observed for prochloraz for which the weaker effect was observed with the *MRR1*$^{G583R/G583R}$ mutants.

Substitutions in *ERG11*, *TAC1*, *MRR1*, and *ERG3* have almost no impact on *C. parapsilosis* virulence

We finally took the opportunity of a *G. mellonella* model to assess whether the engineered substitutions may affect the fitness and *in vivo* virulence of *C. parapsilosis* during

TABLE 3 *In vitro* susceptibility of each CRISPR mutants comparatively with their parental strains (*C. parapsilosis* ATCC 22019 and STZ5) to fluconazole and three other triazoles determined by gradient concentration strips[a,b]

Aminoacid substitutions	Minimal inhibitory concentrations (µg/mL) to azoles according to each background							
	Fluconazole		Voriconazole		Posaconazole		Isavuconazole	
	ATCC 22019	STZ5	ATCC 22019	STZ5	ATCC 22019	STZ5	ATCC 22019	STZ5
None (background strain)	1.5 (S)	0.38 (S)	0.023 (S)	0.023 (S)	0.032	0.032	0.023	0.032
ERG11$^{Y132F/WT}$	**6 (SDD)**	**4 (SDD)**	0.125 (S)	**0.19 (I)**	0.023	0.023	0.032	0.032
ERG11$^{Y132F/Y132F}$	**24 (R)**	**8 (R)**	**0.25 (I)**	**0.38 (I)**	0.016	0.023	0.032	0.032
ERG11$^{K143R/WT}$	**4 (SDD)**	**3 (SDD)**	0.047 (S)	0.064 (S)	0.032	0.047	0.023	0.032
ERG11$^{K143R/K143R}$	**8 (R)**	**8 (R)**	0.094 (S)	0.064 (S)	0.047	0.047	0.032	0.047
ERG11$^{R398I/R398I}$	0.75 (S)	0.38 (S)	0.016 (S)	0.023 (S)	0.023	0.032	0.023	0.032
TAC1$^{G650E/G650E}$	**6 (SDD)**	**3 (SDD)**	0.125 (S)	0.047 (S)	0.125	0.047	0.094	0.047
TAC1$^{L978W/L978W}$	**12 (R)**	**6 (SDD)**	0.064 (S)	0.094 (S)	0.094	0.094	0.094	0.064
MRR1$^{G583R/WT}$	**32 (R)**	–[c]	**0.25 (I)**	–[c]	0.064	–[c]	0.125	–[c]
MRR1$^{G583R/G583R}$	**>256 (R)**	**>256 (R)**	**1.5 (R)**	**3 (R)**	0.094	0.38	0.19	0.25
ERG3$^{G111R/G111R}$	0.5* (S)	0.125* (S)	0.012* (S)	0.016* (S)	0.012*	0.032*	0.006*	0.012*

[a]S: Sensible; SDD: Sensible Dose-Dependent; I: Intermediate; R: Resistant.
[b]Non -susceptible MICs (i.e., above the susceptibility breakpoint, when available) are depicted in bold face.
[c]"–": mutant not available (MIC not performed).

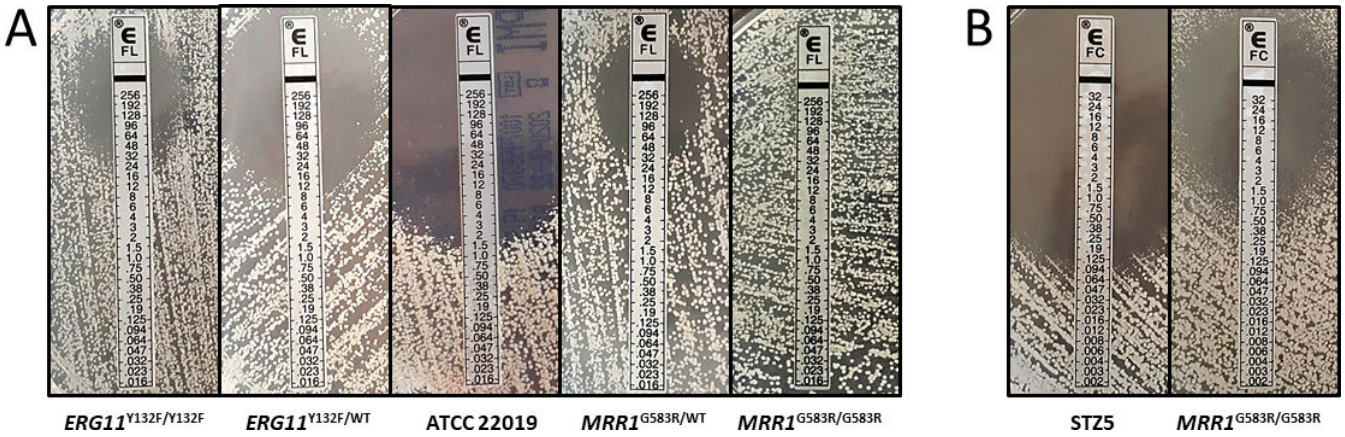

FIG 2 Examples of MIC readings for selected *C. parapsilosis* CRISPR mutants. (A) Influence of the allelic dosage on fluconazole susceptibility illustrated here with Y132F in *ERG11* (left part of the picture) and G583R in the *MRR1* gene (right part of the picture) - *ATCC 22019 background*. (B) The G583R substitution in *MRR1* is associated with an increase in flucytosine MIC (0.75 µg/mL compared with 0.094 µg/mL in the parental strain) - *STZ5 background.*

invasive infection. Overall, according to the Kaplan-Meier analysis, a significant difference in larva survival, between the mutations, independently of the parental strain, was noted (log-rank test $P < 0.05$, panels A and C; Fig. 6). However, the Cox model showed *MRR1*$^{G583R/G583R}$ as the sole substitution associated with a statistically significant effect on larval survival in the ATCC 22019 background. Indeed, larvae infected with the *MRR1*$^{G583R/G583R}$ strain (panel B) displayed improved survival compared with larvae infected with the ATCC 22019 parental strain (HR <1, $P = 2.46 \times 10^{-6}$). Although it did not reach statistical significance, the same trend was observed in the STZ5 background ($P = 0.051$, panel D).

DISCUSSION

Here, we ambitioned to delineate the direct contribution of mutations in *ERG11*, *TAC1*, *MRR1*, and *ERG3* to antifungal resistance in *C. parapsilosis*. We therefore selected seven of the most common amino acid mutations previously reported among

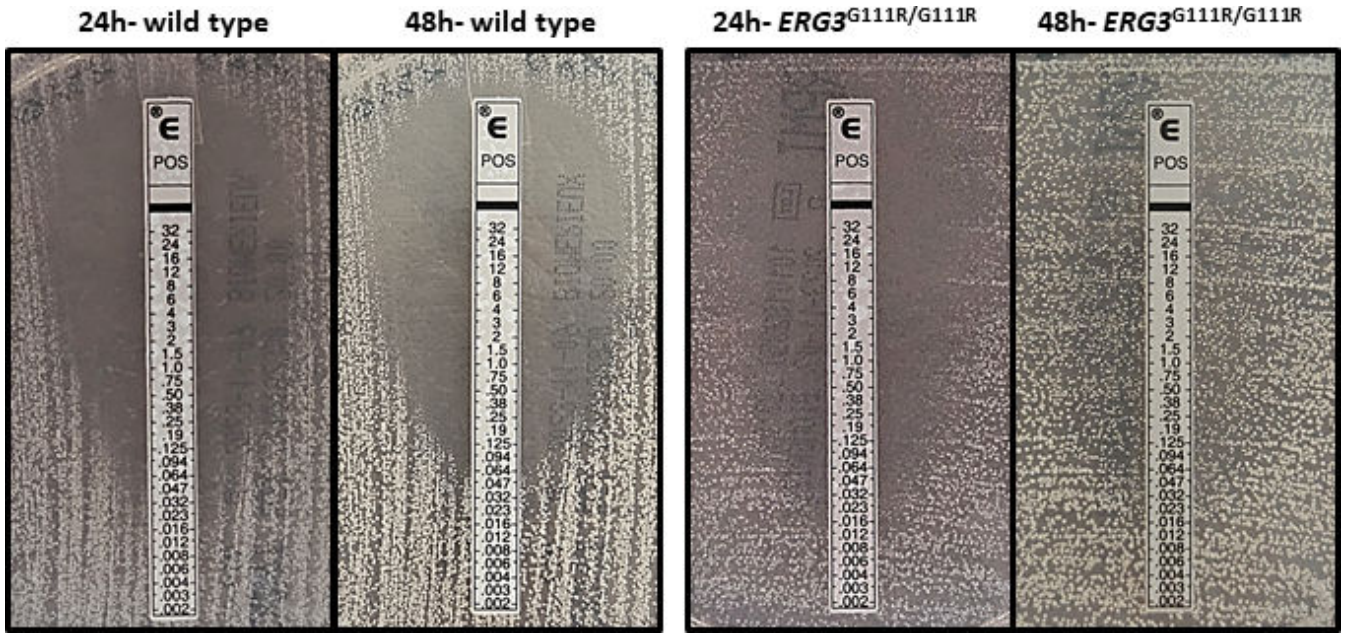

FIG 3 Acquisition of the G111R substitution in *ERG3* confers trailing growth with azoles. Compared with its parental strains (left panel), the G111R mutant (right panel) exhibits a strong trailing growth effect after 48 h incubation with posaconazole. Note that MICs remain unchanged. - *ATCC 22019 background.*

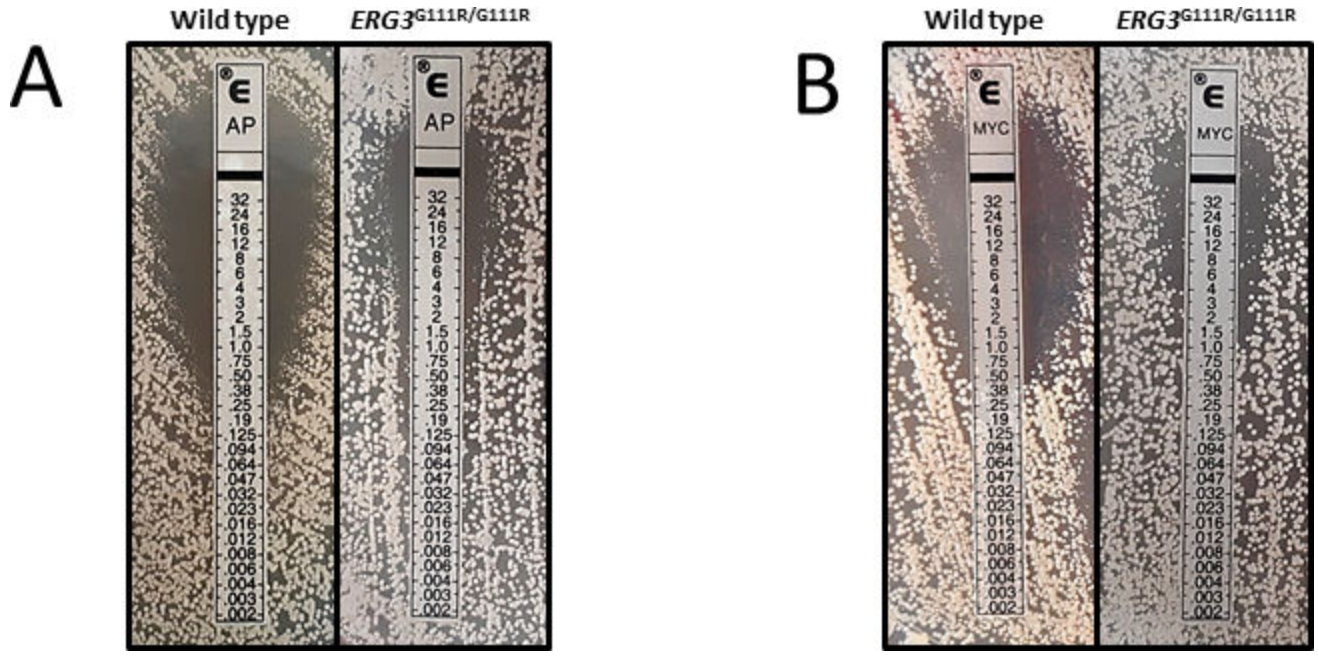

FIG 4 Acquisition of the G111R substitution in *ERG3* alters the susceptibility to non-azole drugs. (A) G111R is responsible for a slight increase in amphotericin B MIC (1.5 µg/mL compared with 0.5 µg/mL in the parental strain) - *ATCC 22019 background.* (B) The same mutation (G111R) also tends to increase micafungin MIC. Note the microcolonies within the inhibition ellipse - *ATCC 22019 background.*

fluconazole-resistant *C. parapsilosis* isolates. To fit our objectives, we took advantage of a CRISPR-Cas9 technique specifically developed for *C. parapsilosis* and allowing both marker-less precise editing and engineering any genetic background, including clinical isolates (24). As different strains of the same species may behave differently as a result of distinct genetic circuitries reflecting unique genetic makeups (27, 28), two different clonal lineages of *C. parapsilosis* were used in this study (ATCC 22019 and STZ5). Furthermore, *C. parapsilosis* being a diploid species, a trait able to mitigate the response to antifungals (29), we also evaluated the impact of different allelic dosages. We therefore introduced each of the selected mutations, in a single or both alleles of each background strain, and further assessed the *in vitro* susceptibility of the recipient strains to a wide array of antifungals, with a focus on azoles.

Our study shows that the seven amino acid substitutions investigated here, differed in their contribution to fluconazole resistance, cross resistance with other azoles or antifungal classes, and virulence. This finding was well illustrated with the *ERG11* gene for which each of the three investigated substitutions (R398I, Y132F, and K143R) conferred a unique profile. Although R398I mutation did not impact the activity of triazoles, in line

TABLE 4 *In vitro* susceptibility of selected CRISPR mutants comparatively to their parental strains to topical azoles, determined by disk diffusion[a,b]

Amino acid substitutions	Disk diffusion diameters (mm)					
	Econazole (50 µg)		Clotrimazole (50 µg)		Miconazole (50 µg)	
	ATCC 22019	STZ5	ATCC 22019	STZ5	ATCC 22019	STZ5
None (background strain)	30 (S)	30 (S)	34 (S)	34 (S)	23 (S)	25 (S)
ERG11$^{Y132F/Y132F}$	24 (S)	25 (S)	31 (S)	27 (S)	**17 (I)**	**17 (I)**
ERG11$^{K143R/K143R}$	27 (S)	28 (S)	32 (S)	32 (S)	**16 (I)**	**19 (I)**
TAC1$^{L978W/L978W}$	22 (S)	25 (S)	26 (S)	28 (S)	**17 (I)**	**18 (I)**
MRR1$^{G583R/G583R}$	28 (S)	22 (S)	31 (S)	24 (S)	**19 (I)**	**18 (I)**

[a]S: sensible (≥20 mm); I: Intermediate (11–19 mm); R: Resistant (≤10 mm).
[b]Non-susceptible MICs (i.e., above the susceptibility breakpoint) are depicted in bold face.

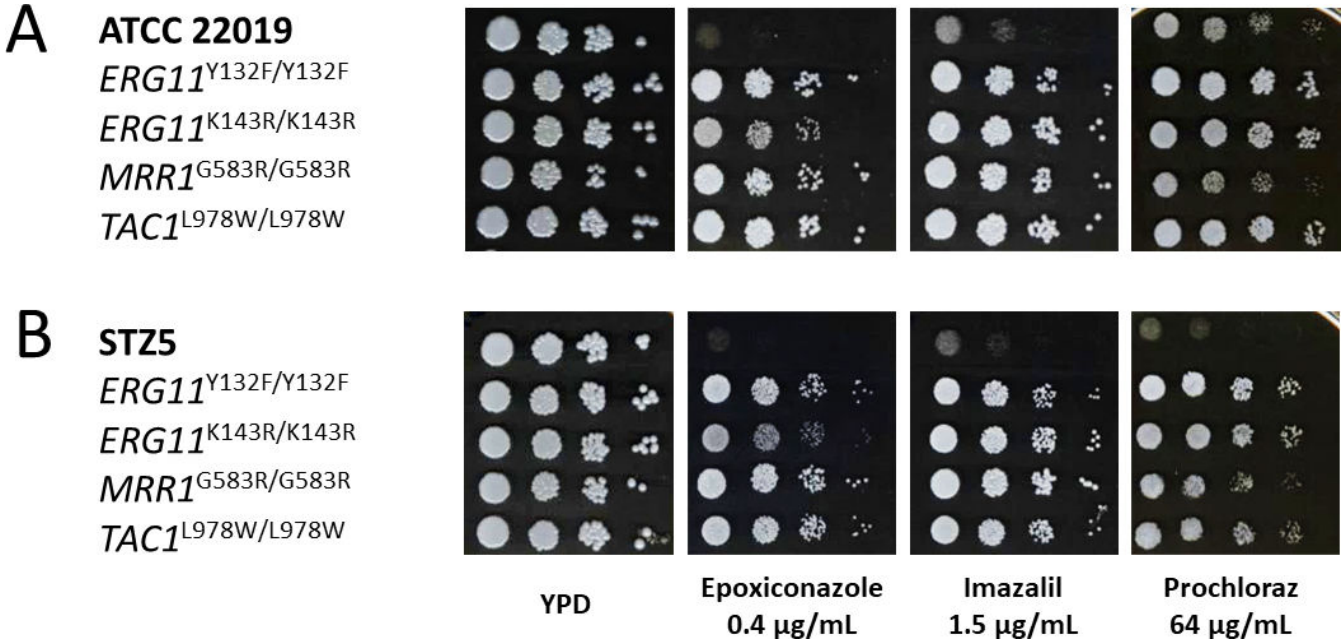

FIG 5 Relative growth of selected mutants compared with their relative parental strains, ATCC 22019 (panel A) or STZ5 (panel B) when exposed to agricultural azoles (epoxiconazole, imazalil, and prochloraz).

with previous studies (13, 30), K143R and Y132F conferred different susceptibility profiles. These residues, both located near the heme-binding site, are expected to reduce the affinity of Erg11p for short-tail triazoles (fluconazole and voriconazole) compared with their long-tail relatives (31). Indeed, we show that both K143R and Y132F impaired fluconazole activity when present in both alleles, whereas posaconazole and isavuconazole MICs were unchanged. However, only Y132F conferred cross-resistance to voriconazole, as previously observed in *Candida auris* (32). Our study also highlights the influence of allelic dosage. In diploid yeasts, a mutation acquired in a single allele can be transferred to the remaining allele through loss of heterozygosity allowing to convert an heterozygous phenotype to an homozygous phenotype (29). This process is part of the genetic evolution of pathogenic yeasts and mitigates the contribution of mutations to antifungal resistance. Despite having a level of heterozygosity much lower than other *Candida* species (33), *C. parapsilosis* strains heterozygous and homozygous for Y132F have been reported in the clinics (16, 34). Here, we demonstrate that the mere presence of Y132F or K143R in a single *ERG11* allele is sufficient to reduce fluconazole susceptibility. This finding is relevant as these substitutions are the most commonly observed in fluconazole-resistant *C. parapsilosis* causing clonal outbreaks worldwide (6, 35). It is worth noting that Y132F has also been reported in several other opportunistic yeasts including *C. albicans*, *Candida tropicalis*, *Candida orthopsilosis*, and *C. auris* as well as in more distant species such as *Diutina catenulata* and *Yarrowia lipolytica*, showing its central role in fluconazole resistance (23, 36–38). Recently, Doorley et al. reported a high incidence of Y132F in a large collection of *C. parapsilosis* from the US (19) but suggested its modest contribution to fluconazole resistance. Here, we demonstrate that the contribution of Y132F to antifungal resistance is dependent both on its allelic dosage and the genetic background of the *C. parapsilosis* strain. Taken together, these findings suggest that clinical strains showing high fluconazole MICs should be investigated for substitutions in other genes.

Besides *ERG11*, we provide additional data on the impact of amino acid substitutions in other genes and primarily in two transcription factors encoding genes namely *TAC1* and *MRR1*. Our findings support the role of G650E and L978W as gain-of-function mutations leading to *TAC1* hyperactive alleles. Although G650E has been previously

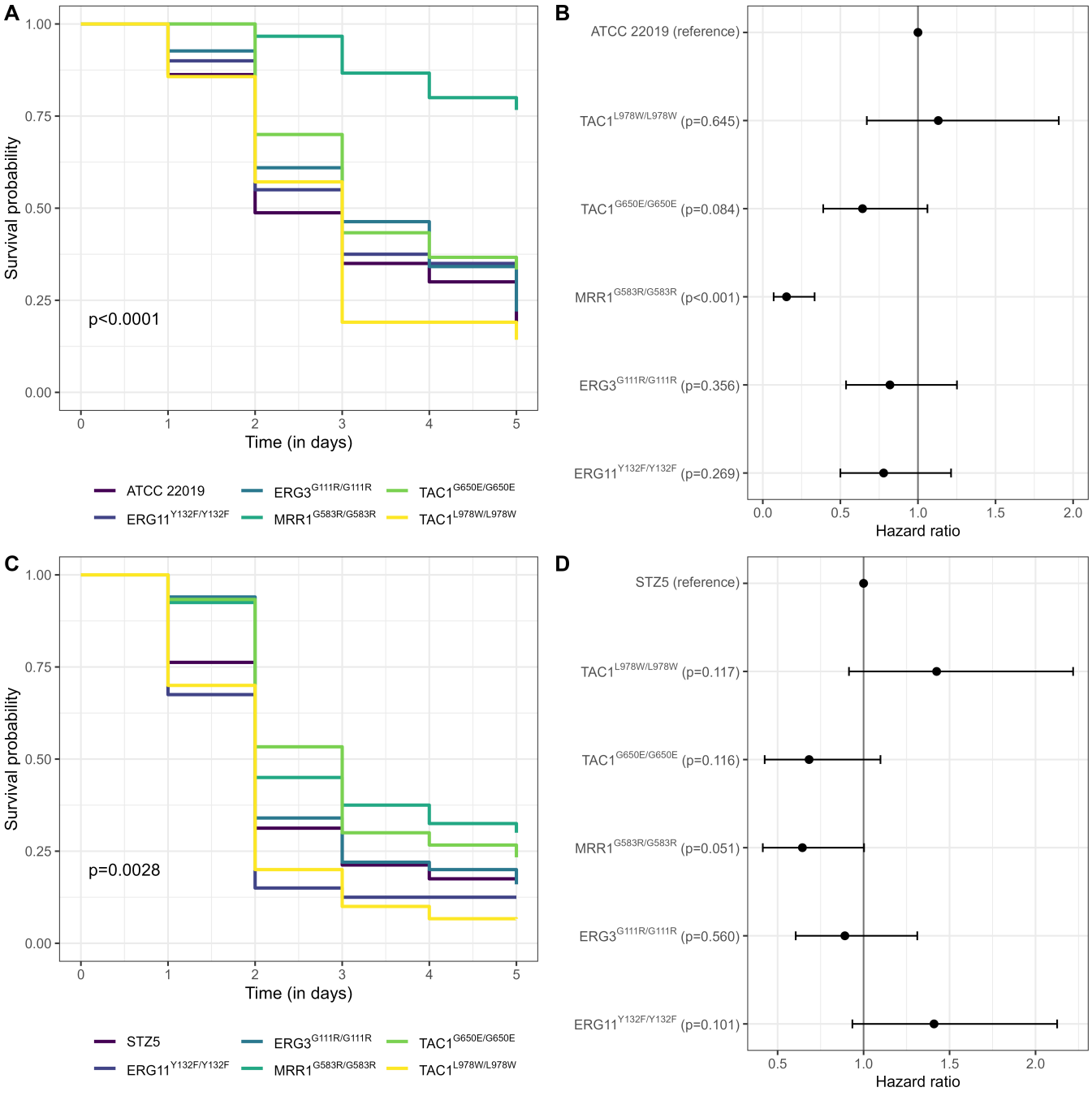

FIG 6 *In vivo* virulence of selected mutants compared with their relative parental strains, ATCC 22019 (panel A and B) and STZ5 (panel C and D), in a *G. mellonella* model. Panel A and C: Kaplan-Meier survival curves. The *P* value refers to the overall difference between all the survival curves drawn on the corresponding panel determined by the log-rank test. Panel B and D: Cox model hazard ratios and their confidence intervals for each larvae group infected by mutants, according to the genetic background (ATCC 22019 and STZ5). The *P* values refer to pairwise comparison of the death incidence between the corresponding mutation and the parental strain. Footnotes: Total number of larvae for each strain included in the experiments: ATCC 22019 genetic background: parental strain (n = 80), *ERG11*$^{Y132F/Y132F}$ (n = 40), *ERG3*$^{G111R/G111R}$ (n = 41), *MRR1*$^{G583R/G583R}$ (n = 30), *TAC1*$^{G650E/G650E}$ (n = 30), and *TAC1*$^{L978W/L978W}$ (n = 21). STZ5 genetic background: parental strain (n = 80), *ERG11*$^{Y132F/Y132F}$ (n = 40), *ERG3*$^{G111R/G111R}$ (n = 50), *MRR1*$^{G583R/G583R}$ (n = 40), *TAC1*$^{G650E/G650E}$ (n = 30), and *TAC1*$^{L978W/L978W}$ (n = 30).

associated with the overexpression of the ABC-transporters *CpCDR1*, *CpCDR1B*, and *CpCDR1C* (19), to our knowledge, it is still unknown whether every gain-of-function mutation confers a similar *TAC1* hyperactivation. Interestingly, according to our data, G650E seems to have a weaker effect on fluconazole resistance than L978W, but whatever the *TAC1* substitution considered, the activity of voriconazole, posaconazole,

and isavuconazole remained unchanged. We also demonstrated the pivotal role of *MRR1* to resistance. Considering the strong contribution of G583R to fluconazole resistance, it is reasonable to suggest a role for this mutation as a predictor of clinical failure (39). Beyond fluconazole, G583R was the only substitution able to affect the MIC of all azoles, although its impact on isavuconazole remains difficult to establish in the absence of Epidemiological Cut-Off Value (ECV). Recently, additional *MRR1* gain-of-function mutations have been reported (16, 22). A few years ago, V668G was reported as a gain-of-function in *MRR1* conferring fluconazole resistance in *Clavispora lusitaniae* (40). Strikingly, V668G was associated with reduced flucytosine susceptibility, in relation to the upregulation of the multidrug transporter *MFS7* (also referred as *MDR1*). This unexpected finding prompted us to investigate the flucytosine susceptibility of our *C. parapsilosis* strains carrying the G583R. Here, and despite the genetic distance between *C. lusitaniae* and *C. parapsilosis*, we also evidenced a link between gain-of-function mutations in *MRR1* and reduced flucytosine susceptibility in *C. parapsilosis*. Considering that *MRR1* hyperactive alleles lead to the upregulation of *CpMDR1*, *CpMDR1B*, and *CpCDR1B* in *C. parapsilosis*, it is likely that one of them may also efflux flucytosine (22).

Impairment of C-5 desaturase activity that leads to the so-called *ERG3* mutant phenotype has also been reported in *C. parapsilosis* (21, 41). A common characteristic of this phenotype, mostly studied in *C. albicans*, is a tendency toward higher MICs to amphotericin due to a reduced ergosterol membrane content (42). The contribution of *ERG3* substitutions to fluconazole has long been debated, between trailing growth and true resistance. Some authors demonstrated that this phenotype *in C. albicans* is actually unique and distinct from trailing growth and azole resistance and further showed distinct species-specific difference in outcome when exposed to azoles (43, 44). A few years ago, Rybak et al. described the G111R substitution as responsible for *ERG3* impairment in *C. parapsilosis* (21). Strikingly, the authors suggested that this mutation was also responsible for micafungin resistance, in addition to amphotericin B and fluconazole. As we show here, this unexpected phenomenon is more likely to be caused by increased tolerance to micafungin than true resistance. Whether this collateral effect is unique to *C. parapsilosis* or shared by other yeasts remains to be determined.

Finally, to further expand our view and correlation mutations with azole resistance, we challenged our mutant strains with various other azole-based compounds namely topic imidazoles and agricultural azoles. Indeed, azole antifungals are notorious for their dual use in both human/veterinary medicine and agriculture, which explains the development of resistance in human fungal pathogens, especially *Aspergillus fumigatus* (45). Of note, *in vitro* exposure of *C. parapsilosis* to agricultural azoles was previously reported to cause resistance to medical azoles (46). We show here that resistance to medical azoles may also lead to cross resistance to agricultural azoles. Although an environmental resistance route may also exist in yeasts, as discussed recently (47–49), our findings suggest that environmental azole-resistant isolates may also have their origin in the clinical setting through selective pressure with medical azoles.

We finally underline that except for *MRR1*, acquisition of mutations in *ERG11*, *TAC1*, and *ERG3* do not seem to come at a cost for *C. parapsilosis*, suggesting that fluconazole-resistant strains may have a similar fitness than their susceptible relatives. This finding may explain the potential of fluconazole-resistant strains to rapidly spread from patient to patient within clinical wards and the community as observed in Spain (34, 35). Our study however has some limitations as we only investigated one of the most common substitutions identified in the clinics and did not include *UPC2* nor *FKS* mutations which result in an incomplete overview of the molecular mechanisms behind antifungal resistance in *C. parapsilosis.* Future works will aim to include more mutations, alone or in combinations, as antifungal resistance can evolve in a stepwise manner.

Altogether, we provide evidence that mutations in *ERG11*, *TAC1*, *MRR1*, and *ERG3* do not uniformly lead to acquired resistance to all azole compounds. Some of them are able to confer high-level resistance to fluconazole, suggesting that they can be used to predict the risk of fluconazole failure. Of note, we show that posaconazole and

isavuconazole may remain active against some fluconazole-resistant strains suggesting a possible added value of these antifungals for the management of patients with refractory infections. Hopefully, the efficiency of genome editing using CRISPR-Cas9 and its rapid spread in research labs will facilitate the elucidation of the *C. parapsilosis* resistome and will pave the way for the development of next-generation sequencing pipelines allowing a rapid identification of mutations associated with antifungal resistance (6, 50).

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ADDITIONAL FILES

The following material is available online.

Supplemental Material

Table S1 (AAC00022-24-S0001.docx). Primers used in this study.

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