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Fluconazole and Voriconazole Resistance in *Candida parapsilosis* Is Conferred by Gain-of-Function Mutations in *MRR1* Transcription Factor Gene

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***Candida parapsilosis* is the second most prevalent fungal agent causing bloodstream infections. Nevertheless, there is little information about the molecular mechanisms underlying azole resistance in this species. Mutations (G1747A, A2619C, and A3191C) in the *MRR1* transcription factor gene were identified in fluconazole- and voriconazole-resistant strains. Independent expression of *MRR1* genes harboring these mutations showed that G1747A (G583R) and A2619C (K873N) are gain-of-function mutations responsible for azole resistance, the first described in *C. parapsilosis*.**

Candida parapsilosis is a human fungal pathogen causing infections that range from mucocutaneous to systemic. Interestingly, *C. parapsilosis* infections display an unusual prevalence in terms of age and geographical distribution. Invasive fungal infections caused by *C. parapsilosis* afflict mainly young patients (neonates and children), surpassing in number those caused by *Candida albicans*. Geographically, *C. parapsilosis* is the second most common agent of fungal infection in Mediterranean, South American, and Asian countries (1–3). Studies regarding the drug susceptibility profile of *C. parapsilosis* isolates have been carried out worldwide, and apart from their reduced susceptibility to echinocandins (4), their level of resistance to azoles has not been alarming so far (5–7). However, with the extensive use of azoles as therapeutic and prophylactic drugs prescribed in *C. parapsilosis* infections, it is expected that azole resistance will emerge more frequently. This concern was recently the focus in a study of *C. parapsilosis* fluconazole surveillance carried out in clinical isolates from several hospitals in the United States (6). Fluconazole susceptibility was assessed in 706 *C. parapsilosis* isolates, collected in 80 hospitals. Interestingly, fluconazole resistance rates vary from 70%, in isolates found in Atlanta hospitals, to 0%, in isolates coming from Portland, OR. The study of the molecular mechanisms underlying fluconazole resistance revealed the first mutations described in clinical isolates, either in the *MRR1* transcription factor or in the azole target, the *ERG11* gene (6).

We have demonstrated that *in vitro* exposure to azoles, namely, fluconazole, voriconazole, and posaconazole, triggers a stable development of azole resistance in *C. parapsilosis* (8). Interestingly, exposure to posaconazole induced cross-resistance to other azoles, while fluconazole and voriconazole induced only cross-resistance to each other (9). Azoles act by inhibiting an enzyme involved with the ergosterol biosynthesis pathway, the enzyme 14-α-demethylase, encoded by *ERG11*. The lack of ergosterol production and/or the production of toxic metabolites as a result of Erg11p inactivation seriously disrupts fungal growth. Fungal cells can overcome these effects by overexpressing *ERG11*, reducing azole affinity to its target molecule through mutation, or expelling the azole from the intracellular to the extracellular environment (10). The efficiency of this last strategy requires overexpression of efflux pumps, encoded by the ABC transporter superfamily (*CDR1*, *CDR2*) or the major facilitator superfamily (*MDR1*) (11).

The fact that fluconazole and voriconazole induce cross-resistance in *C. parapsilosis* suggests that the underlying mechanisms are similar. This is supported by comparison of the transcriptional profiles of fluconazole- and voriconazole-resistant strains, which showed that two genes associated with acquisition of resistance were overexpressed, *MDR1* (multidrug resistance) and its regulator, the transcription factor *MRR1* (multiresistance regulator) (9). Two different missense mutations (G1747A and A2619C) were identified in the *MRR1* genes from fluconazole- and voriconazole-resistant strains, leading to alterations in the Mrr1p polypeptide chain from glycine to arginine (G583R) and from lysine to asparagine (K873N), respectively (9). Subsequent analysis of *MRR1* from the voriconazole-resistant isolate detected an additional mutation, A3191C, causing a change from glutamine to a proline (Q1064P) in Mrr1p. Other *MRR1* mutations were recently described in fluconazole-resistant clinical isolates; however, their impact in fluconazole resistance was not conclusive, since the same polymorphisms were also found in susceptible clinical isolates, with the one exception being the G2337T mutation (6).

Extensive characterization in *C. albicans* and *C. glabrata* has shown that gain-of-function mutations in transcription factors, like *MRR1*, *TAC1*, *UPC2*, or *PDR1*, are involved in the develop-

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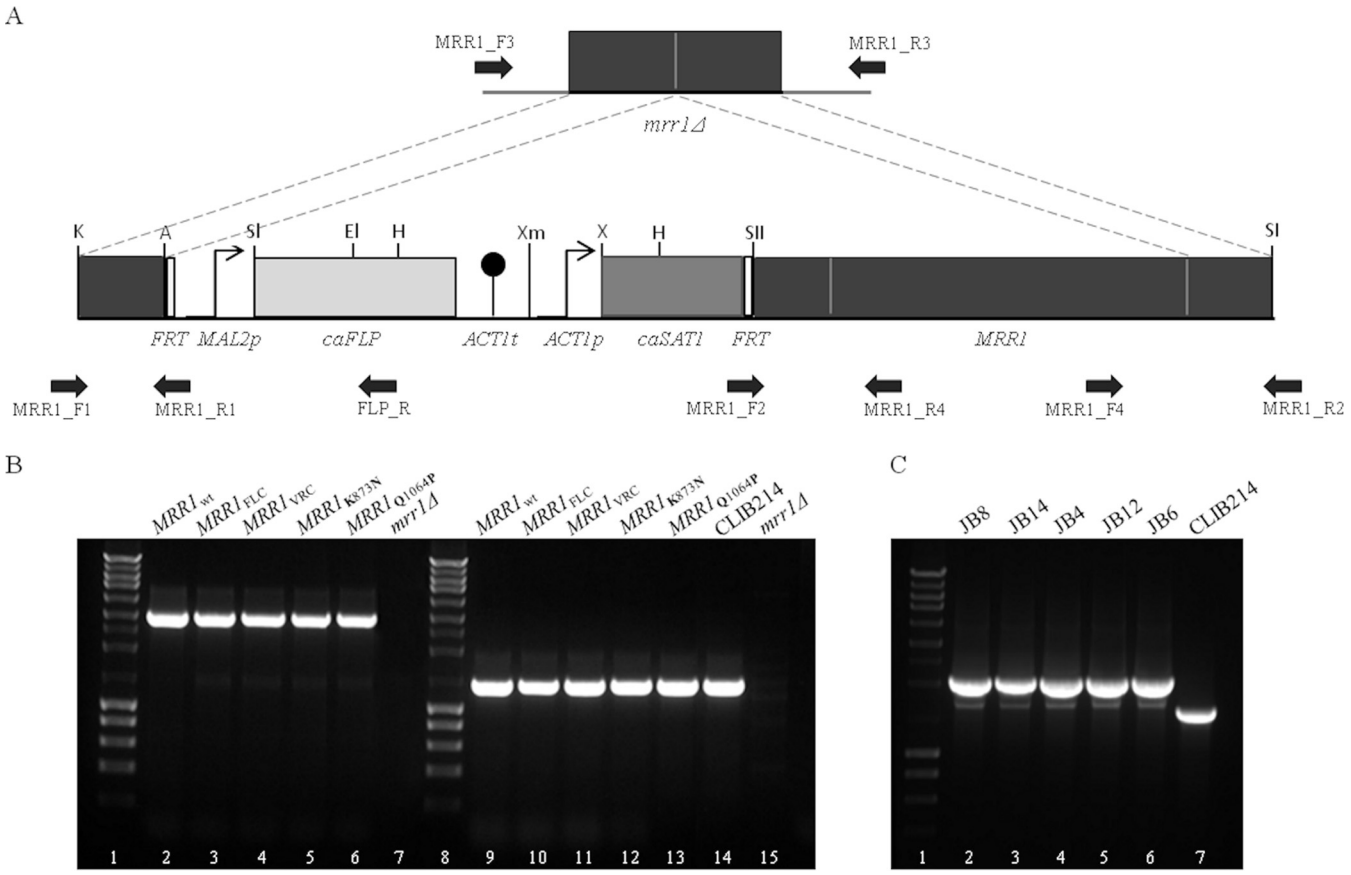

FIG 1 Genomic integration of the *MRR1* transcription factor gene variants in the *C. parapsilosis* homozygous disruptant *mrr1*Δ strain. (A) *MRR1* complementation cassettes were constructed using the *SAT1* flipper cassette (20) and primers MRR1_F1, MRR1_R1, MRR1_F2, and MRR1_R2 (Table 1). $MRR1_{wt}$, MRR_{FLC} (containing mutation G1747A), MRR_{VRC} (containing mutations A2619C and A3191C), $MRR1_{K873N}$ (containing mutation A2619C), and $MRR1_{Q1064P}$ (containing mutation A3191C) genes were integrated in the *C. parapsilosis mrr1*Δ double mutant, generating the JB8, JB14, JB4, JB12, and JB6 strains, respectively. Each mutation in MRR_{VRC} was independently silenced by site-directed mutagenesis (Table 1) to originate $MRR1_{K873N}$ and $MRR1_{Q1064P}$. (B) Complementation cassettes were integrated at the *MRR1* native genomic locus and confirmed by PCR using the following pairs of primers: (i) MRR1_F3 and FLP_R, which amplified a 3-kb fragment (lanes 2 to 6; absent in lane 7), and (ii) MRR1_F4 and MRR1_R3, resulting in a 1.3-kb PCR product (lanes 9 to 13). Lanes 14 and 15 show a 1.3-kb fragment present in CLIB214 and absent in the *mrr1*Δ strain, respectively. Lanes 1 and 8 represent the molecular size marker (NZYDNA Ladder III). (C) Confirmation of the successful recycling of the *MRR1* complementation cassettes by PCR using primers MRR1_F3 and MRR1_R4, resulting in the amplification of a 2.1-kb fragment (lanes 2 to 6), CLIB214 was used as control amplifying a 1.7-kb fragment (lane 7). The discrepancy between size fragments amplified in the transformants and the one amplified in CLIB214 is due to the presence of two promoters regions upstream of the *MRR1* gene in the transformants compared with only one in the CLIB214 strain. Lane 1 represents the molecular size marker (NZYDNA Ladder III).

ment of azole resistance in these species (12–19). These mutations activate the transcription factors, which constitutively upregulate their targets: efflux pumps or ergosterol biosynthesis enzymes.

To determine if G583R, K873N, and Q1064P in *MRR1* are gain-of-function mutations associated with fluconazole and voriconazole resistance in *C. parapsilosis sensu stricto*, a set of constructs was generated using the *SAT1* flipper cassette (Fig. 1A) (20). Five cassettes were constructed, representing $MRR1_{wt}$ (wild-type gene) (GenBank accession number KT160017), $MRR1_{FLC}$ (with the G583R mutation) (GenBank accession number KT160018), $MRR1_{VRC}$ (with K873N and Q1064P mutations) (GenBank accession number KT160019), $MRR1_{K873N}$ (with the K873N mutation only), and $MRR1_{Q1064P}$ (with the Q1064P mutation only). These were introduced into a *C. parapsilosis* strain with the two copies of the endogenous *mrr1*Δ gene deleted (21). After electroporation (20), transformants were selected in yeast extract-peptone-dextrose (YPD) agar plates containing 200 μg/ml nourseothricin. Integration of the cassettes at the native *MRR1* locus was confirmed by PCR (Fig. 1B). All primer sequences are listed in Table 1. After recycling of the *SAT1* flipper (Fig. 1C), the azole susceptibility profile and expression of *MRR1* and *MDR1* in each strain was determined.

Azole susceptibility profiles of the strains expressing $MRR1_{wt}$, $MRR1_{FLC}$, $MRR1_{VRC}$, $MRR1_{K873N}$, $MRR1_{Q1064P}$, the *mrr1*Δ strain, and the parent of the *mrr1*Δ mutant (*C. parapsilosis* CLIB214) were characterized according to CLSI protocols (M27-S4 and M27-A3) (22, 23). The expression of $MRR1_{FLC}$ (strain JB14), $MRR1_{VRC}$ (strain JB4), and $MRR1_{K873N}$ (strain JB12) conferred resistance to fluconazole and voriconazole (Table 2). In contrast, strains expressing $MRR1_{Q1064P}$ (JB6) or $MRR1_{wt}$ (JB8) and the parent CLIB214 and *mrr1*Δ strains are susceptible to the same antifungal drugs (Table 2). This demonstrates that missense mutations G1747A (G583R) and A2619C (K873N) in *MRR1* determine resistance to fluconazole and voriconazole in *C. parapsilosis*, whereas Q1064P has no effect. As expected, no alteration of susceptibility phenotype was found for posaconazole (Table 2).

TABLE 1 Primers used in this study

Primer name	Primer sequence (5′ to 3′)
Construction of cassettes	
MRR1_F1	GGGGGTACCCTACTGATATGCCTGACGCCAC
MRR1_R1	GGGGGGCCCTCTCTCTTATTGAAAACAAGAAAGC
MRR1_F2	TCCCCGCGGCTACTGATATGCCTGAGGCCAC
MRR1_R2	GGGGAGCTCTCTCTCTTATTGAAAACAAGAAAGC
Site-directed mutagenesis	
MRR1VSDM_F1	CGAGGTATTTTTACGCATGGAAATTGACAAAGAGTCATTCTTATT
MRR1VSDM_R1	AATAAGAATGACTCTTTGTCAATTTCCATGCGTAAAAATACCTCG
MRR1VSDM_F2	GCGGCCCCAGCAACAACAGCCTATAGGG
MRR1VSDM_R2	CCCTATAGGCTGTTGTTGCTGGGGCCGC
PCR confirmation	
MRR1_F3	GAAAACAAGTAATCAAAACACGGGG
FLP_R	TTTATGATGGAATGAATGGGATG
MRR1_F4	CGGCATCTCGCAGCAACAA
MRR1_R3	GTCTGTAAAGGGGGGGGTTGGA
MRR1_R4	ACTTGAACGAAATGGAGACC
qRT-PCR	
TUB4_F	TGTATTCCACAATGATGCCT
TUB4_R	TGCCTTGAAACGAAGTAGC
MRR1_F	ACAATGGTCTGAGCAATGAA
MRR1_R	GGCAATACTGGTGATGGAA
MDR1_F	TTCGTGATAGTTTTGGTGGTAG
MDR1_R	TGAACCTGGAGTGAATCTTGT

To elucidate the mechanism of action, expression of *MRR1* and *MDR1* was determined by quantitative real-time PCR (qRT-PCR) (Table 1). The expression levels of the $MRR1_{FLC}$, $MRR1_{VRC}$, and $MRR1_{K873N}$ alleles were 3- to 4-fold higher than those of the $MRR1_{Q1064P}$ and $MRR1_{wt}$ alleles (Fig. 2). The effect on *MDR1* expression is more dramatic; expression was up to 70 times higher in strains expressing $MRR1_{FLC}$ (strain JB14), $MRR1_{VRC}$ (JB4), and $MRR1_{K873N}$ (JB12) than in CLIB214 or strains expressing $MRR1_{Q1064P}$ (JB6) or $MRR1_{wt}$ alleles (JB8) (Fig. 2). Therefore, only G1747A and A2619C, but not A3191C, confer hyperactivity to the *MRR1* transcription factor, which consequently upregulates the *MDR1* multidrug transporter. Thus, G583R and K873N mutations are the first gain-of-function mutations described in the *C. parapsilosis MRR1* transcription factor.

In a large surveillance study of fluconazole-resistant clinical isolates of *C. parapsilosis* in the United States, the most common underlying molecular mechanisms were associated with polymorphisms in *ERG11* and *MRR1* (6). According to these authors, polymorphisms in *MRR1* are common, and only some are associated with overexpression of *MDR1*. They suggest that there is a hot spot for gain-of-function mutations in *MRR1*, in the region coding from amino acids 852 to 875, which is equivalent to a similar region in *MRR1* in *C. albicans* and *C. dubliniensis* (6, 14). They identified one clinical isolate with a polymorphism in this region, corresponding to L779F, which has 73-fold upregulation of *MDR1* (6). Our results corroborate this finding since the K873N mutation is located in this so-called gain-of-function hot spot region but also show that mutations located in other regions (G583R) are equally important for azole resistance. Strains expressing G583R and K873N *MRR1* mutants upregulate *MDR1* expression to a level similar to that of the L779F isolate (around 70-fold increase compared to that of the wild type) (6), conferring resistance to fluconazole and voriconazole (MIC, ≤64 μg/ml). Grossman et al. identified four other *MRR1* putative gain-of-function polymorphisms present in fluconazole-resistant clinical isolates (6). However, they did not conclusively show that polymorphisms in *MRR1* cause increased expression of *MDR1*, as they also identified *MRR1* polymorphisms in susceptible isolates.

TABLE 2 MIC values and susceptibility phenotypes of *C. parapsilosis* strains

Strain	MIC (μg/ml)/phenotype[a] for:		
	Fluconazole	Voriconazole	Posaconazole
CLIB214	1/S	0.03/S	0.06/WT
*mrr1*Δ mutant	1/S	0.03/S	0.06/WT
$MRR1_{wt}$ (JB8)	1/S	0.03/S	0.06/WT
$MRR1_{FLC}$ (JB14)	64/R	2/R	0.12/WT
$MRR1_{VRC}$ (JB4)	64/R	2/R	0.12/WT
$MRR1_{K873N}$ (JB12)	64/R	2/R	0.12/WT
$MRR1_{Q1064P}$ (JB6)	1/S	0.03/S	0.06/WT

[a] S, susceptible; R, resistant; WT, wild type.

Other mechanisms of azole resistance, such as *ERG11* mutations, should also be considered. For example, the A395T (Y132F) polymorphism was found in more than 50% of 30 fluconazole-resistant clinical isolates of *C. parasilosis* (6).

In conclusion, we described the first gain-of-function mutations, G583R and K873N, in the *C. parapsilosis MRR1* transcription factor involved in fluconazole and voriconazole resistance. Furthermore, these data confirm our previous suggestion (9) that *MDR1* is a key player in fluconazole and voriconazole resistance in *C. parapsilosis*.

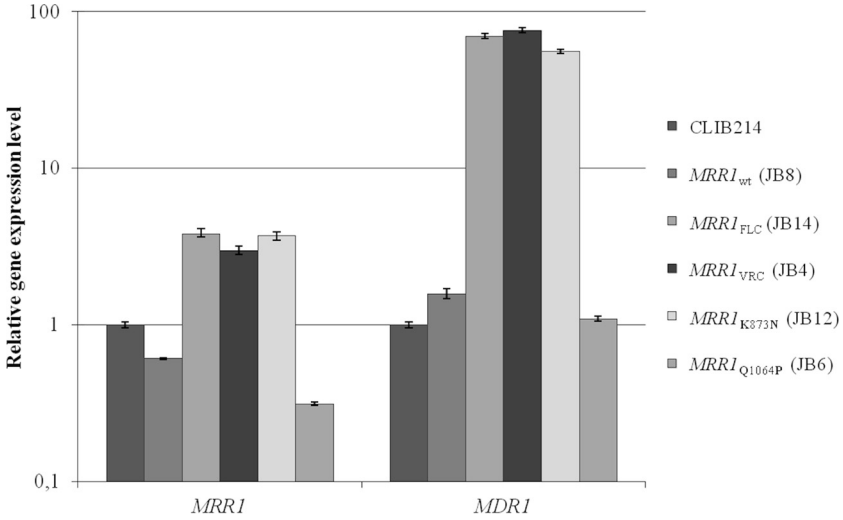

FIG 2 Mutations G583R and K873N induce overexpression of *MRR1* and *MDR1* genes. Quantitative real-time PCR (qRT-PCR) analysis of the *MRR1* and *MDR1* gene expression in the JB8, JB14, JB4, JB12, and JB6 strains, using the CLIB214 strain as a control. Due to the large range in gene expression levels, the *y* axis scale is logarithmic. The expression values displayed in the graph represent the variation of *MRR1* and *MDR1* gene expression relative to the CLIB214 strain and are expressed as the mean of five independent experiments, with the respective standard deviation. Amplification efficiency and the amount of *TUB4* (endogenous reference gene) were used to normalize each mean value.

Nucleotide sequence accession numbers. Sequences have been deposited in GenBank under the accession numbers KT160017, KT160018, and KT160019.

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