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MECHANISMS OF RESISTANCE

A Novel Combination of *CYP51A* Mutations Confers Pan-Azole Resistance in *Aspergillus fumigatus*

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ABSTRACT The treatment of invasive and chronic aspergillosis involves triazole drugs. Its intensive use has resulted in the selection of resistant isolates, and at present, azole resistance in *Aspergillus fumigatus* is considered an emerging threat to public health worldwide. The aim of this work is to uncover the molecular mechanism implicated in the azole resistance phenotype of three *Aspergillus fumigatus* clinical strains isolated from an Argentinian cystic fibrosis patient under long-term triazole treatment. Strain susceptibilities were assessed, and *CYP51A* gene sequences were analyzed. Two of the studied *Aspergillus fumigatus* strains harbored the TR34-L98H allele. These strains showed high MIC values for all tested triazoles (>16.00 μg/ml, 1.00 μg/ml, 1.00 μg/ml, and 2.00 μg/ml for itraconazole, isavuconazole, posaconazole, and voriconazole, respectively). The third strain had a novel amino acid change (R65K) combined with the TR34-L98H mutations. This new mutation combination induces a pan-azole MIC augment compared with TR34-L98H mutants (>16 μg/ml, 4.00 μg/ml, 4.00 μg/ml, and 8.00 μg/ml for itraconazole, isavuconazole, posaconazole, and voriconazole, respectively). The strain harboring the TR34-R65K-L98H allele showed no inhibition halo when voriconazole susceptibility was evaluated by disk diffusion. The effect of these mutations in the azole-resistant phenotype was confirmed by gene replacement experiments. Transformants harboring the TR34-L98H and TR34-R65K-L98H alleles mimicked the azole-resistant phenotype of the clinical isolates, while the incorporation of the TR34-R65K and R65K alleles did not significantly increase azole MIC values. This is the first report of the TR34-L98H allele in Argentina. Moreover, a novel *CYP51A* allele (TR34-R65K-L98H) that induces a pan-azole MIC augment is described.

KEYWORDS Argentina, *Aspergillus*, *CYP51A*, South America, TR34/L98H, azole, mutation, resistance

Fungal colonization of the respiratory tract is frequent in cystic fibrosis patients. However, its impact in lung deterioration is still not clear (1). During recent years, it has become apparent that *Aspergillus fumigatus* may play a role in the pathogenesis of lung function declination (2, 3). Its persistence is associated with several and diverse clinical presentations, such as bronchitis and allergic bronchopulmonary aspergillosis, that might lead to an accelerated loss of pulmonary function (4).

The treatment of aspergillosis in cystic fibrosis patients typically involves a primary therapy with triazole drugs. These antifungals target fungal 14-α sterol demethylases,

Citation Macedo D, Brito Devoto T, Pola S, Finquelievich JL, Cuestas ML, Garcia-Effron G. 2020. A novel combination of *CYP51A* mutations confers pan-azole resistance in *Aspergillus fumigatus*. Antimicrob Agents Chemother 64:e02501-19. https://doi.org/10.1128/AAC.02501-19.

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Received 17 December 2019
Returned for modification 13 February 2020
Accepted 25 April 2020
Accepted manuscript posted online 18 May 2020
Published 22 July 2020

TABLE 1 Nucleotide and amino acid substitutions in the *cyp51A* gene and MICs for the *A. fumigatus* clinical isolates included in this study[a]

		CYP51A gene and promoter substitution			MIC (μg/ml) of:						
Isolate	5′ UTR	Residue 65	Residue 98	Residue 297	VRC[d]	ITC	POS	ISA	AMB	CAS	AFG
A	TR34	AGA (R)	CAC (H)	ACG (T)	2.00* (10)	>16.00*	1.00	1.00	0.50	0.12	0.06
B	TR34	AAA (K)	CAC (H)	ACG (T)	8.00* (0)	>16.00*	4.00*	4.00	0.50	0.12	0.06
C	TR34	AGA (R)	CAC (H)	ACG (T)	2.00* (10)	>16.00*	1.00	1.00	0.50	0.12	0.06
LMDM-31	WT	AGA (R)	CTC (L)	TCG (S)	0.12 (32)	0.12	0.25	0.50	0.25	0.06	0.03
LMDM-32	WT	KO	KO	KO	0.03 (48)	0.03	0.06	0.06	0.25	0.06	0.03
LMDM-1427[b]	TR34	AGA (R)	CAC (H)	TCG (S)	2.00* (10)	>16.00*	1.00	1.00	0.25	0.06	0.03
LMDM-1428[b]	TR34	AAA (K)	CAC (H)	TCG (S)	8.00* (0)	>16.00*	4.00*	4.00	0.25	0.06	0.03
LMDM-1599[c]	TR34	AAA (K)	CTC (L)	TCG (S)	0.12 (31)	0.25	0.25	0.50	0.25	0.06	0.03
LMDM-1600[c]	WT	AAA (K)	CTC (L)	TCG (S)	0.12 (31)	0.25	0.25	0.50	0.25	0.06	0.03

[a]5′ UTR, 5′ untranslated region; WT, wild type; TR34, 34-bp tandem repetition; VRC, voriconazole; ITC, itraconazole; POS, posaconazole; ISA, isavuconazole; AMB, amphotericin B; CAS, caspofungin; AFG, anidulafungin; *, considered non-wild type by the published epidemiological cutoff values (17, 18).
[b]LMDM-31 was used as the recipient strain.
[c]LMDM-32 was used in transformation experiments. This strain was obtained using LMDM-31 (21).
[d]Numbers in parentheses are inhibition diameters in mm.

key enzymes in the biosynthetic pathway of ergosterol. These enzymes are encoded in *Aspergillus* spp. by two paralog genes named *CYP51A* and *CYP51B* (5, 6).

The increased use of triazole drugs has resulted in the selection of resistant strains, and at present, azole resistance in *A. fumigatus* is considered an emerging threat to public health worldwide (7). Two routes of azole resistance development have been described in *A. fumigatus*. First, resistant strains may be selected during or after a long-term azole therapy in the context of chronic pulmonary aspergillosis, such as it occurs in cystic fibrosis patients (8). Second, azole resistance may be related to the extended use of azole antifungals in agriculture. These environmental azole-resistant *A. fumigatus* strains colonize azole-naive patients (9–12).

The most prevalent mechanism of azole resistance in *A. fumigatus* involves mutations in the *CYP51A* gene (13, 14). Among them, the most frequently reported mutations are those involving the residues G54, G138, G448, and M220 together with the alleles TR34-L98H and TR46-Y121F-T289A (14). The first three mutations are thought to have emerged during triazole therapy in the clinical setting, whereas those containing tandem repeats (TRs) in the promoter region of the *CYP51A* gene are thought to have been selected due to azole fungicide use in agriculture. Substitutions at the residue M220 were mostly detected after azole treatment but also in azole naive patients (possible environmental acquisition) (9, 10, 15, 16).

In the present study, we describe three azole-resistant *A. fumigatus* clinical strains isolated from different sputum samples collected from an Argentinian cystic fibrosis patient. Two of them carry the well-known TR34-L98H allele (16), and they are the first clinical strains with these mutations described in Argentina. The third strain showed a novel amino acid substitution (R65K) combined with the already mentioned TR34-L98H mutations. This newly described mutation combination induces a pan-azole MIC augment. The effect of this new mutation on the azole-resistant phenotype was confirmed by gene replacement experiments.

RESULTS

Strain identification, clonality, and antifungal susceptibility testing. Based on their calmodulin (*CaM*) gene sequences, the three isolates recovered from one cystic fibrosis patient were identified as *A. fumigatus sensu stricto*. To investigate whether these strains were different, their multilocus sequence type (MLST) patterns were established (17). Strains A and B (obtained from sputum samples in October 2015 and November 2016, respectively) shared the same MLST pattern (ANX4, allele 7; BGT1, allele 1; CAT1, allele 3; LIP, allele 2; MAT1-2, allele 1; SODB, allele 2; ZRF2, allele 1), while strain C (recovered from the patient in June 2017) showed a different one (ANX4, allele 1; BGT1, allele 1; CAT1, allele 3; LIP, allele 1; MAT1-2, allele 5; SODB, allele 1; ZRF2, allele 2). These strains exhibited reduced susceptibility to triazole drugs (Table 1). Considering

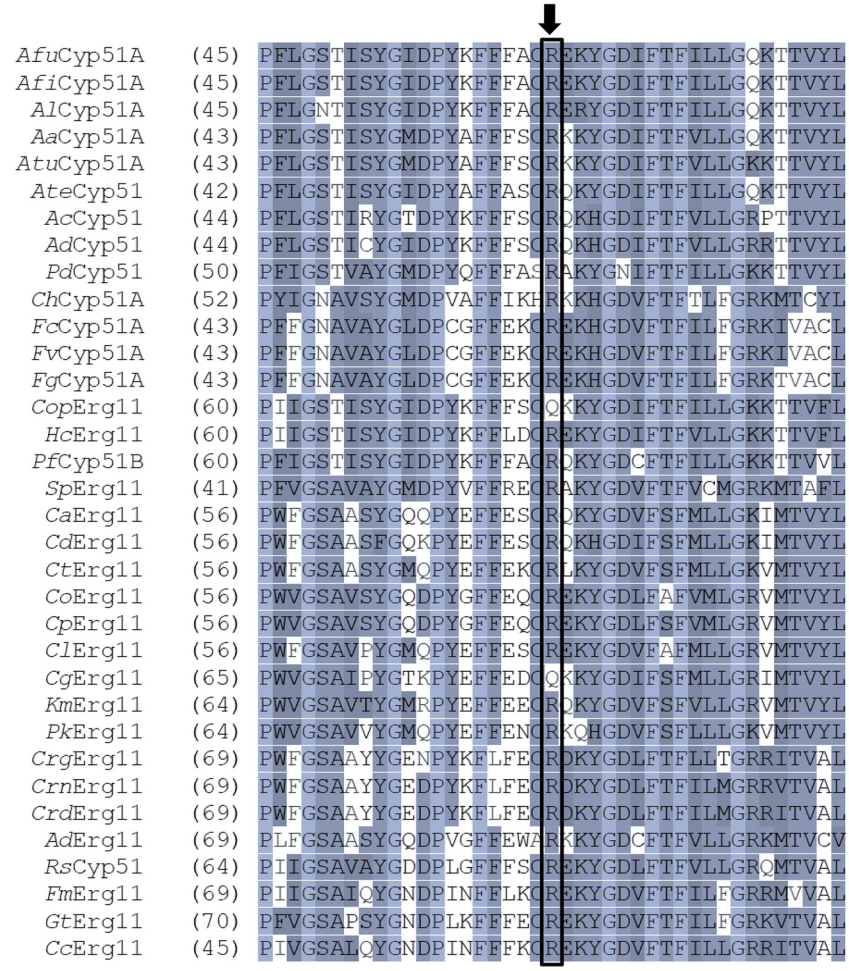

FIG 1 Clustal W alignment of a fragment of different Cyp51p/Erg11p. AfuCyp51A, *A. fumigatus* Cyp51Ap (AF338659.1); *Afi*Cyp51A, *Aspergillus fischeri* (XP_001267338.1); *Al*Cyp51A, *Aspergillus lentulus* (ADI80344.1); *Aa*Cyp51A, *Aspergillus awamori* (AEK81588.1); *Atu*Cyp51A, *Aspergillus tubingensis* (AEK81587.1); *Ate*Cyp51A, *Aspergillus terreus* (XP_001215095.1); AcCyp51, *Ajellomyces capsulatus* (EEH10950.1); *Ad*Cyp51, *Ajellomyces dermatitidis* (EGE84227.1); *Pd*Cyp51, *Penicillium digitatum* (CAD27793.1); *Ch*Cyp51A, *Colletotrichum higginsianum* (CCF38358.1); *Fc*Cyp51A, *Fusarium cerealis* (AFN66168.1); *Fv*Cyp51A, *Fusarium vorosii* (AFN66167.1); *Fg*Cyp51A, *Fusarium graminearum* (ACL93387.1); *Cop*Erg11, *Coccidioides posadasii* (AAU01157.1); *Hc*Erg11, *Histoplasma capsulatum* (AAU01158.1); *Pf*Cyp51B, *Pseudocercospora fijiensis* (XP_007928752.1); *Sp*Erg11, *Schizosaccharomyces pombe* (CAA90803.1); *Ca*Erg11, *Candida albicans* (P10613.2); *Cd*Erg11, *Candida dubliniensis* (AAK57519.1); *Ct*Erg11, *Candida tropicalis* (AMR44154.1); *Co*Erg11, *Candida orthopsilosis* (CCG24173.1); *Cp*Erg11, *Candida parapsilosis sensu stricto* (SBU87529.1); *Cl*Erg11, *Candida lusitaniae* (ACH87138.1); *Cg*Erg11, *Candida glabrata* (AMR44146.1); *Km*Erg11, *Kluyveromyces marxianus* (AHL25033.1); *Pk*Erg11, *Pichia kudriavzevii* (ABI54469.1); *Crg*Erg11, *Cryptococcus gattii* (AEQ63274.1); *Crn*Erg11, *Cryptococcus neoformans* (AAP12370.1); *Crd*Erg11, *Cryptococcus neoformans* (AAF35366.1); *Ad*Erg11, *Auricularia delicata* (EJD49069.1); RsCyp51, *Rhizoctonia solani* (CCO28946.1); *Fm*Erg11, *Fomitiporia mediterranea* (EJD06923.1); *Gt*Erg11, *Gloeophyllum trabeum* (EPQ54358.1); *Cc*Erg11, *Coprinopsis cinerea* (AAU01159.1). GenBank accession numbers appear after the species names in parenthesis. The alignment was performed using Vector NTI Advance (v11.5.4) software. The black box and arrow denote the arginine (R) residue under study.

the published epidemiological cutoff (ECOFF) values (18, 19), all strains were considered non-wild type for itraconazole and voriconazole (>16 and MICs ranging from 2.00 to 8.00 μg/ml, respectively), while strain B was non-wild type for all the tested azole drugs (isavuconazole and posaconazole; 4.00 μg/ml) (Table 1). When voriconazole susceptibility was established using agar diffusion, strains A and C showed a 10-mm inhibition diameter, while strain B showed no inhibition at all (Table 1; Fig. 1). On the other hand, the strains showed low amphotericin B, caspofungin, and anidulafungin MIC values (Table 1).

Sequence analysis of *CYP51A* genes and their promoters. The three studied strains showed two point mutations at their *CYP51A* open reading frames, namely, t364a and t960a. These mutations were responsible for the amino acid substitutions L98H and S297T, respectively. In addition, the three strains also contained a 34-bp tandem duplication sequence in the *CYP51A* promoter (between positions −288 and −322). Strain B had an extra base change at *CYP51A* (a194g), leading to the substitution R65K (Table 1). Clustal alignment of different Cyp51Ap/Erg11p sequences showed that the R65 residue is conserved in a great diversity of fungal species, including yeast, molds, and dimorphic ascomycetes and several basidiomycetes. On the other hand, important pathogenic fungal species, such as *Candida glabrata* and *Coccidioides posadasii*, harbor a polymorphism (arginine by glutamine) at their Cyp51Ap/Erg11p site at the equivalent position (Fig. 1).

***CYP51A* gene replacement.** To establish the implication of the newly observed *CYP51A* mutation combination on triazole MIC increases, we initially transformed by electroporation the azole-susceptible *A. fumigatus* LMDM-31 strain (akuB$_{KU80}$Δ) (20, 21) with four linear PCR fragments named TR34-65K-98H, TR34-98H, TR34-65K, and 65K. These fragments carry the promoter alteration plus the substitutions at the 65 and 98 residues (1.1 kb), the 34-bp promoter repetition together with the substitution at the 98 residue (1.1 kb), the promoter alteration and the substitution at position 65 (2.2 kb), and the mutation that confers the R65K substitution alone (1.6 kb), respectively.

First, transformations were performed using strain LMDM-31 as the recipient strain and itraconazole 1 μg/ml was used as the selector. Homologous recombination and integration of two of the PCR fragments (TR34-98H and TR34-65K-98H) were obtained, and the integration was confirmed phenotypically by PCR and by sequencing in several mutants ($n = 5$ for TR34-98H and $n = 4$ for TR34-65K-98H) (Fig. 2). These transformants were obtained in two separate transformation experiments performed in different weeks. On the other hand, and after eight transformation experiments, no transformants were recovered when TR34-65K and 65K PCR fragments were intended to be introduced into LMDM-31 conidia by transformation. Thus, we decided to use an azole-hypersusceptible strain (*A. fumigatus* LMDM-32) as the recipient strain (21, 22) and using lower itraconazole concentrations (0.12 μg/ml) as the selector. Doing so, we tried to establish if subtle azole MIC shifts were produced by this newly described mutation. Using these new conditions, transformants were obtained with the other two PCR fragments (TR34-65K and 65K).

Studied transformants were named LMDM-1427 (LMDM-31::TR34-L98H), LMDM-1428 (LMDM-31::TR34-R65K-L98H), LMDM-1599 (LMDM-32::TR34-R65K), and LMDM-1600 (LMDM-32::R65K). These transformants were subjected to antifungal susceptibility testing by microdilution and by agar diffusion. LMDM-1427 and LMDM-1428 strains showed the same triazole susceptibility patterns as the clinical strains A (and C) and B, respectively. Both transformant strains showed a 266-fold increase in itraconazole MICs compared with the parental strain (LMDM-31). On the other hand, the transformant harboring the TR34/R65K/L98H allele (LMDM-1428) had a 4-fold increase in isavuconazole, posaconazole, and voriconazole MICs compared with the already azole-resistant (itraconazole/voriconazole) LMDM-1427 mutant strain (TR34/L98H) (Table 1). The reduction in voriconazole susceptibility was also evident for LMDM-1428 when the susceptibility was evaluated by using disk diffusion. The inhibition zone was reduced from 32 mm (parental LMDM-31 strain) to 10 mm (LMDM-1427) and was inexistent for LMDM-1428 (TR34-R65K-L98H) (Fig. 3). The described mutant phenotypes exactly mimicked the phenotypes of the clinical strains. On the other hand, transformants that do not harbor the L98H substitution (LMDM-1599 and LMDM-1600) showed slight or no azole MIC increases (Table 1).

DISCUSSION

Several mutations in the coding region of the *A. fumigatus CYP51A* gene associated or not with sequence repetitions in its promoter have been found to confer different patterns of azole resistance in *A. fumigatus* (14). The most commonly described

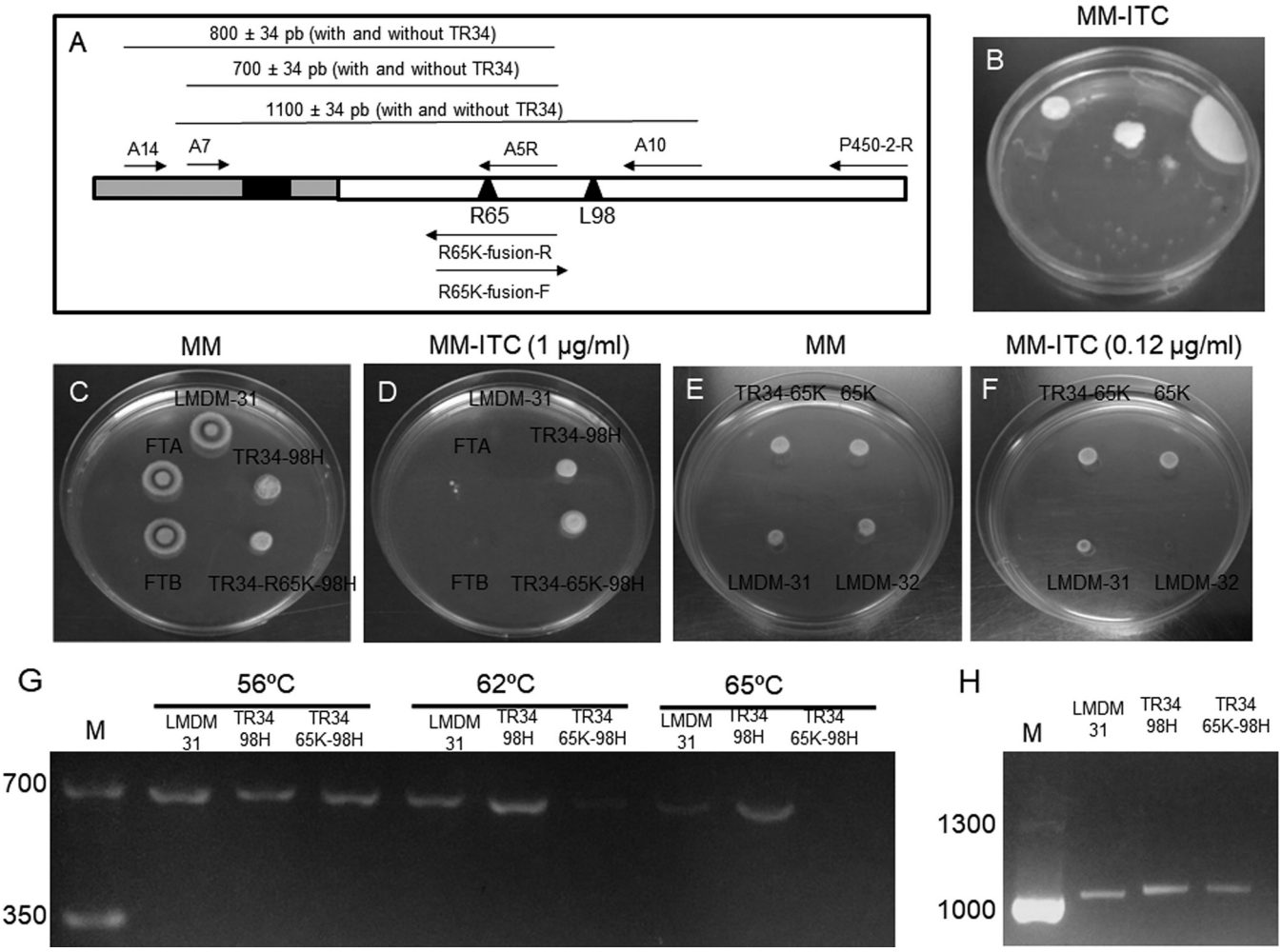

FIG 2 (A) Representation of the relative positions of the primers used in this work. Gray and white boxes symbolize the 5′ UTR and the ORF of the AfCYP51A gene, respectively. The black box and arrows represent the 34-bp sequence that is repeated in resistant strains (TR34) and the used primers, respectively. Thin lines symbolize the expected PCR band sizes.(B) Selection plates of minimal medium plus itraconazole (1 μg/ml) (MM-ITC) where the transformant LMDM-1428 (LMDM-31::TR34-65K-98H) was obtained. (C to F) Replica plates used minimal medium (MM) (C and E) and minimal medium plus itraconazole (1 μg/ml) (MM-ITC) and plus itraconazole (0.12 μg/ml) (D and F, respectively). Some false transformants (isolated during the transformation process but with no transformation cassette integration) were included in photography and named FTA and FTB (C and D). (G) Homologous recombination of the TR34-65K-98H fragment was confirmed by using gradient PCRs and A7/A5R primers. Lane M, ladder (numbers on the left are in nucleotides). PCR amplification using DNA from parental strain LMDM-31 (lanes 1, 4, and 7), LMDM-1427 (LMDM-31::TR34-98H) (lanes 2, 5, and 8), and LMDM-1428 (LMDM-31::TR34-65K-98H) (lanes 3, 6, and 9). (H) Confirmation of the integration of the TR34 sequence in the promoter of the *CYP51A* gene using a PCR with A7/A10 primers. Lane M, ladder (numbers express the size in nucleotide). Lane 1, LMDM-31; lane 2, LMDM-1427 (LMDM-31::TR34-98H); lane 3, LMDM-1428 (LMDM-31::TR34-65K-98H).

resistance allele is TR34-L98H. It was first described in Spanish and Dutch *A. fumigatus* isolates and later worldwide (14–16). In South America, this combination of *CYP51A* alterations was described in isolates obtained from flower fields in Colombia and from azole-naive patients in Peru, confirming the idea that such strains are potentially propagated by the use of fungicides in agriculture (demethylase inhibitors [DMIs]) (23, 24). In this work, we present the first *A. fumigatus* strains (strains A and C) harboring this allele isolated in Argentina (7). Our isolates also carry the S297T substitution at Cyp51Ap. In 2007, Mellado et al. described an *A. fumigatus* strain carrying the same allele (TR34-L98H-S297T) (16). They demonstrated that only TR34-L98H is enough and necessary to confer the complete azole resistance phenotype (16). Later, it was suggested that the extra S297T mutation might represent a compensatory mutation (25). Oppositely, our third strain (named B strain) carries an R65K Cyp51Ap substitution (associated with the TR34-L98H-S297T) that confers a pan-azole MIC increase (including posaconazole and isavuconazole) and a complete insensitivity to voriconazole when tested by agar diffusion (no inhibition halo observed). The effect of the R65K mutation

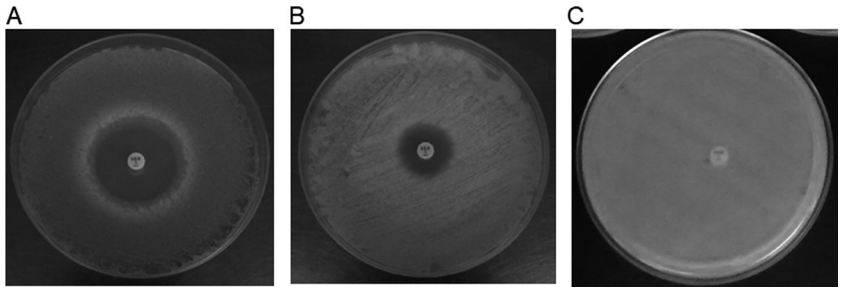

FIG 3 Susceptibility evaluation by agar diffusion and voriconazole paper disks for *A. fumigatus* parental strain (LMDM-31) (A), *A. fumigatus* transformant strain named LMDM-1427 (TR34-L98H) (B), and *A. fumigatus* transformant strain named LMDM-1428 (TR34-R65K-L98H) (C).

on the described phenotype was confirmed in this work by gene replacement experiments. We used different PCR fragments in order to individualize the effect of the newly described Cyp51Ap substitution. The obtained transformants showed that the integration of the TR34-L98H allele reproduced the results published earlier (16), but it was not sufficient to reproduce the pan-azole MIC increase and the voriconazole insensitivity when tested by agar diffusion. On the other hand, the introduction of TR34-R65K-L98H allele into the *A. fumigatus* LMDM-31 strain produces a phenotype that exactly mimicked the MIC values observed in the clinical *A. fumigatus* strain (strain B). In addition, we also confirmed that the sole R65K mutation or that associated with the TR34 promoter alteration does not produce important azole susceptibility modifications. Similar results were obtained by Mellado et al. (16) when the sole L98H substitution or the TR34 promoter alteration were transformed into an azole-susceptible *A. fumigatus*. Thus, it seems that the association of these two *CYP51A* mutations together with the TR34 promoter are needed to confer the azole resistance phenotype. The three *A. fumigatus* strains studied herein were isolated from a cystic fibrosis patient who was receiving a long-term triazole therapy. The patient received itraconazole that was switched later to voriconazole. Strains A and B were isolated in the course of itraconazole, while strain C was cultured during voriconazole therapy. The acquisition of TR34-L98H-mediated resistance appears to be related to the so-called environmental route of azole resistance acquisition (10). This concept implies that patients are infected by strains that acquired their resistance in the environment (they are already resistant at the time of the infection) and not by susceptible strains that become resistant during treatment. Knowing these facts and adding the MLST results, we can speculate that our patient was infected by two different environmental strains carrying the TR34-L98H-S297T (A and C) allele that were selected during itraconazole and voriconazole treatment, respectively. The origin of strain B is not so clear since it shared the same MLST pattern with strain A but only the B isolate harbors the newly described allele (TR34-R65K-L98H-S297T). However, MLST has enough discriminatory power to establish if two strains are different but not to determine if two isolates are clonal (26).

This fact makes it difficult for us to explain how the R65K mutation emerged during itraconazole therapy and why it was not isolated later during voriconazole treatment even when it is more resistant to the latest. One possible rationalization is the random isolation of each of the strains in a context of a nonclonal (mixed) infection, as suggested by Kolwijck et al. (27). However, further studies are needed to establish the route of acquisition of the R65K mutation.

The results presented here, together with other recent papers, demonstrate that *A. fumigatus* triazole resistance in Argentina is emerging (28, 29).

MATERIALS AND METHODS

Samples, strains, and media. Three fungal isolates recovered from different sputum samples of an Argentinean cystic fibrosis patient obtained in October 2015, November 2016, and June 2017 (identified as A, B, and C, respectively) were sent to the Centro de Micología, Instituto de Microbiología y

Parasitología Médica, Universidad de Buenos Aires-CONICET. The patient received itraconazole treatment (400 mg/day) during 2015 to 2016. In 2017, antifungal treatment was switched to voriconazole (200 mg/day). Isolates were identified to section level by macroscopic and microscopic morphology and to species level by sequencing of the *CaM* gene (30). *A. fumigatus* ATCC 204305 and *Aspergillus flavus* ATCC 204304 were used as quality-control strains for susceptibility testing. *A. fumigatus* LMDM-31 (akuB$_{KU80}$Δ) (20) and *A. fumigatus* LMDM-32 (LMDM-31:: CYP51AΔ) were used as recipient strains in electroporation assays (21).

PCR amplification, clonality, and sequencing of the *CYP51A* and *CaM* genes. Conidia from each strain were inoculated into GYEP broth (2% glucose, 0.3% yeast extract, and 1% peptone), and DNA extraction was performed as previously described (5). *CaM* gene amplification was performed using the set of primers CMD5 and CMD6 (30). The full sequences of the *CYP51A* genes and its 5′ untranslated region (UTR) and 3′ UTR regions were amplified (31), and sequences were analyzed using the Unipro UGENE v1.29.0 software (32). *CYP51A* sequences were aligned with wild-type *A. fumigatus CYP51A* (GenBank accession number AF338659). The three clinical isolates underwent multilocus sequence typing (MLST) using the Bain et al. MLST scheme (17) and the *Aspergillus* PubMLST (https://pubmlst.org/cgi-bin/mlstdbnet/mlstdbnet.pl?page=allseq&file=af_profiles.xml).

***Aspergillus fumigatus* transformation vectors, transformation method, and integration confirmation.** *A. fumigatus* LMDM-31 was transformed by electroporation with two linear DNA fragments of 1.1 kb long each, following a method published earlier (16, 21, 22). One of the fragments was named TR34-L98H and contained the *cyp51A* promoter sequence of *CYP51A* with the 34-bp tandem repeat in combination with a t364a mutation (leading to a L98H substitution). The second fragment was designated TR34-R65K-L98H and included the same promoter and open reading frame (ORF) mutations described for the fragment TR34-L98H but with a second nucleotide change (g194a) that generates the substitution R65K. Both fragments were amplified by PCR using the primer set A7-A10 and the genomic DNAs from *A. fumigatus* A and B strains. Primer A7 (5′-TCATATGTTGCTCAGCGG -3′) hybridizes to the AfCYP51A 5′ UTR 462 nucleotides (nt) downstream of the *CYP51A* start codon, and primer A10 (5′-GGACATCTCTGCGGCAAT-3′) hybridizes 604 nt upstream of the start codon. Transformants were selected on minimal medium using itraconazole (1 μg/ml).

Strain LMDM-32 was transformed with two different linear PCR fragments (2.1 kb and 1.6 kb) obtained by fusion PCR and cloned later into two pGEM-T easy vectors (Promega, Biodynamics Argentina). Vectors were named TR34-65K and 65K. The last vector (*cyp51A* with the a194g mutation) was generated by amplifying the DNA obtained from LMDM-31 and introducing the a194g mutation by fusion PCR. The first two PCR amplifications were done using the following primer pairs: A7 together with R65K-fusion-R (5′-CTACAATCTTGAGACTTGCCTTTTCcCTGCACGCAAAGAAGAACTTGTAGG-3′) and R65K-fusion-F (5′-CCTACAAGTTCTTCTTTGCGTGCAGgGAAAAGGCAAGTCTCAAGATTGTAG-3′) together with P450-2-R (21) (5′-CTGTCTCACTTGGATGTG-3′) (lowercase letters indicate the inclusion of the nucleotide a194g change). Later, these two fragments (682 nt and 1,449 nt, respectively) were fused by PCR using the A7 and P450-2-R primers and cloned into a pGEM-T easy vector.

The transformation vector TR34-65K was generated using DNA obtained from the clinical strain A and the vector 65K. The first DNA was used to PCR amplify a 692-nt *CYP51A* fragment (496 nt downstream from the start codon of the *CYP51A* to 220 nt upstream of the ATG, including a TR34 duplication) using the primers A7 and R65K-fusion-R. The vector 65K was used as the DNA template to amplify a second portion of the *CYP51A* gene (220 nt to 1,619 nt) by using the R65K-fusion-F together with P450-2-R (5′-CTGTCTCACTTGGATGTG-3′) (21). These fragments were fused using the A7 and P450-2-R primers and cloned into a pGEM-T easy vector.

An itraconazole resistance phenotype was expected after the incorporation of a functional mutant *cyp51a* gene. The integration of the DNA fragments was phenotypically confirmed by plating the strains in duplicate in minimal medium and minimal medium containing 1 μg/ml and 0.12 μg/ml itraconazole to select transformants with LMDM-31 and LMDM-32 as the genetic background, respectively. Genomic DNA from itraconazole-resistant transformants and parental strains was obtained. Incorporation of the cassettes in the *A. fumigatus* genome was screened by a gradient PCR (temperature [T] annealing from 56°C to 66°C), using primers A7 and A5R (5′-CTTCTTTGCGTGCAGAGA-3′). Primer A5R hybridizes 197 nt upstream of the start codon of AfCYP51A. The mutation R65K (g194a nucleotide change) produced a negative PCR result since primer A5R would not hybridize at the upper limit of the T annealing gradient (Ta, >65°C). On the other hand, another PCR was designed to assess the presence of a *cyp51a* promoter alteration using the primers A7 and A10. If the TR fragments were incorporated in the genome, a 1,100-nt PCR band would be observed. In contrast, a 1,066-nt PCR fragment would be obtained for the parental strain or for nontransformant strains. PCR amplifications were performed in an Applied Biosystems thermocycler (Tecnolab-AB, Argentina) in a 25-μl final volume by using Pegasus DNA polymerase (PBL, Argentina). The cycling program used included an initial 2-min step at 95°C, followed by 30 cycles of 30 s at 95°C, 30 s at the melting temperature of the primer set used (56°C for A7/A10 and a gradient from 56°C to 66°C for A7/A5R to assess the presence of the g194a mutation), and 90 s at 72°C. A final cycle of 10 min at 72°C was included. The *CYP51A* genes, including the promoter area from the transformants, were sequenced to confirm their incorporation.

Antifungal susceptibility testing. Antifungal susceptibility testing by microdilution was performed following the CLSI M38 3rd ed. document (33). Amphotericin B, anidulafungin, caspofungin, itraconazole, isavuconazole, voriconazole, and posaconazole were evaluated. Drugs were obtained as standard powders from their manufacturers or from Merck-Sigma-Aldrich (Argentina). All samples were analyzed at least three times on different days. Susceptibility testing results were interpreted by using the epidemiological cutoff (ECOFFs) values published in the CLSI M59 2nd ed. document for amphotericin B, caspofungin, itraconazole, isavuconazole, and voriconazole (18). For posaconazole, the ECOFFs were

those published by Espinel-Ingroff et al. (19). Voriconazole susceptibilities were also assessed by agar diffusion following the guidelines published in the CLSI document M51A (34).

ACKNOWLEDGMENTS

M.L.C. and G.G.-E. are staff members of CONICET. D.M. and T.B.D. have a PhD fellowship from CONICET (Argentina). D.M. has received an Overseas Scholarship from the British Society for Antimicrobial Chemotherapy (Overseas Scholarship BSAC-2017-0022). This work was supported, in part, by grants from the Ministerio de Ciencia, Tecnología e Inovación Productiva (MinCyT) to M.L.C. (PICT 2016-0206) and to G.G.-E. (PICT 2016-1985).

We declare that we do not have anything to disclose regarding funding or conflict of interest with respect to the manuscript.

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