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CoERG11 A395T mutation confers azole resistance in *Candida orthopsilosis* clinical isolates

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Background: *Candida orthopsilosis* is a human fungal pathogen responsible for a wide spectrum of symptomatic infections. Evidence suggests that *C. orthopsilosis* is mainly susceptible to azoles, the most extensively used antifungals for treatment of these infections. However, fluconazole-resistant clinical isolates are reported.

Objectives: This study evaluated the contribution of a single amino acid substitution in the azole target CoErg11 to the development of azole resistance in *C. orthopsilosis*.

Methods: *C. orthopsilosis* clinical isolates ($n = 40$) were tested for their susceptibility to azoles and their *CoERG11* genes were sequenced. We used a *SAT1* flipper-driven transformation to integrate a mutated *CoERG11* allele in the genetic background of a fluconazole-susceptible isolate.

Results: Susceptibility testing revealed that 16 of 40 *C. orthopsilosis* clinical isolates were resistant to fluconazole and to at least one other azole. We identified an A395T mutation in the *CoERG11* coding sequence of azole-resistant isolates only that resulted in the non-synonymous amino acid substitution Y132F. The *SAT1* flipper cassette strategy led to the creation of *C. orthopsilosis* mutants that carried the A395T mutation in one or both *CoERG11* alleles (heterozygous or homozygous mutant, respectively) in an azole-susceptible genetic background. We tested mutant strains for azole susceptibility and for hot-spot locus heterozygosity. Both the heterozygous and the homozygous mutant strains exhibited an azole-resistant phenotype.

Conclusions: To the best of our knowledge, these findings provide the first evidence that the CoErg11 Y132F substitution confers multi-azole resistance in *C. orthopsilosis*.

Introduction

Severe fungal infections afflict millions of patients, resulting in >1350000 deaths annually,[1] often as a consequence of failure to rapidly treat patients because of delayed or missed diagnosis.[2] *Candida orthopsilosis* is a human fungal pathogen belonging to the *Candida parapsilosis* species complex.[3] These species colonize virtually all body sites and cause a wide spectrum of symptomatic human infections, ranging from mucosal to life-threatening invasive diseases.[4] While *Candida albicans* remains the most frequently isolated *Candida* species,[5] studies conducted in Italy, Spain and Latin America show that the *C. parapsilosis* species complex ranks as the second most common cause of bloodstream infection.[6–8] Although less prevalent than *C. parapsilosis*, the two sibling *C. orthopsilosis* and *Candida metapsilosis* species are often isolated from the blood of paediatric patients, including neonates.[8–12] In this population in particular, the antifungal agent fluconazole is widely used as a prophylactic agent to prevent candidaemia and other forms of invasive candidiasis.[10]

Among the azole resistance mechanisms known to date, genetic modifications of the *ERG11*-encoded lanosterol 14α-demethylase target, resulting in a reduced affinity for the drug, influence the spectrum of azole activities.[11] Fluconazole has the weakest interaction with the Erg11 target and displays the narrowest antifungal spectrum; it is therefore active against yeasts but not against other fungi. As such, fluconazole promotes the broadest antifungal resistance.[11] Of the 70 Erg11 amino acid substitutions described in azole-resistant clinical isolates of *C. albicans*, some result in resistance only to fluconazole, others to voriconazole but not to posaconazole, and some others to all the azole class

members.[12–17] Computational modelling using high-resolution structures as a template may help to explain the impact of specific amino acid substitutions on drug–target interactions in *Candida* species.[18,19] However, due to very low incidence (usually <5%) of *C. orthopsilosis* candidaemia,[20,21] only a small number of studies addressing fluconazole resistance mechanisms are available for this species.[22–26]

In this study, we sought to investigate the molecular mechanisms of azole resistance in *C. orthopsilosis* by using a set of selected azole-susceptible and azole-resistant clinical isolates. To the best of our knowledge, this is the first study demonstrating the role of the A395T *CoERG11* (*C. orthopsilosis ERG11*) mutation in the azole resistance of *C. orthopsilosis*.

Materials and methods

Strains and growth conditions

The *C. orthopsilosis* clinical isolates used in this study were collected at the Microbiology Unit of Pisa University Hospital (Pisa, Italy) and at the Institute of Microbiology of the Università Cattolica del Sacro Cuore, Fondazione Policlinico Universitario Agostino Gemelli (Rome, Italy) (see Table 1). Isolates were obtained from single patients and were identified by MALDI-TOF MS (Bruker Daltonics, Bremen, Germany). Isolates were stored at −80°C in yeast/peptone/dextrose (YPD) broth supplemented with 40% glycerol, and subcultured on YPD agar plates at 30°C when necessary. Prior to use, isolates were grown in YPD broth at 30°C under agitation. For selection of nourseothricin-resistant *C. orthopsilosis* recombinant strains, nourseothricin (Werner BioAgents, Jena, Germany) was added to YPD medium at a 100 mg/L final concentration. For excision of *SAT1* flipper cassette, mutant strains were grown for 48 h at 30°C in 1× yeast nitrogen base (YNB) medium supplemented with 20 g/L maltose. *Escherichia coli* DH5α genotype *F–, endA1, hsdR17 [rk–, mk–], supE44, thi-1, recA1, gyrA96, relA1, Δ[argF-lac]U169*, λ–, *Φ80dlacZΔM15* was used for plasmid construction and propagation. Bacterial cells were grown at 37°C in LB broth or on LB agar plates; when required, ampicillin (Sigma–Aldrich, St Louis, MO, USA) was added to a final concentration of 100 mg/L. All mutant strains were kept in 40% glycerol stock frozen at −80°C.

Antifungal susceptibility testing

C. orthopsilosis clinical isolates and *CoERG11* mutant strains were tested for antifungal susceptibility to fluconazole, posaconazole, itraconazole and voriconazole, in accordance with EUCAST guidelines.[27] MIC values were interpreted using species (*C. parapsilosis*)-specific EUCAST clinical breakpoints (http://www.eucast.org/clinicalbreakpoints/).

CORT_0E05900 sequencing and in silico analysis

Genomic DNA was isolated from *C. orthopsilosis* clinical isolates grown overnight in 10 mL of YPD medium at 30°C as previously described.[28] The *C. orthopsilosis ERG11* gene was identified by searching the http://cgob3.ucd.ie/database, which encompasses the annotated genomes of several clinically relevant *Candida* species, including *C. orthopsilosis* (strain 90-125).[29] Sequencing of the *CORT_0E05900* gene, an orthologue of *C. albicans ERG11* (http://cgob3.ucd.ie/), referred to above as *CoERG11*, was performed using overlapping primers in chromosomal order CORT0E05900f1–CORT0E05900r1 (574 bp, from nucleotide +217 after the STOP codon to +1212), CORT0E05900f2–CORT0E05900r2 (871 bp, from nucleotide +1267 to +396) and CORT0E05900f3–CORT0E05900r3 (984 bp, from nucleotide +588 to −396 before the ATG codon). Primer sequences and parameters used for PCR amplification of specific gene fragments are shown in Table S1 and Figure S1 (available as Supplementary data at *JAC* Online). Gene sequences were deposited in GenBank under accession numbers: MG544857, MG495386, MG495387, MG495388, MG495389, MG495390, MG495385, MG516582, MG544858, MG516583, MG516584, MG543287, MG543288, MG543289, MG601484, MG601487, MG601488, MG601489, MG601490, MG584838, MG584839, MG584840, MG584841, MG584842, MG584843, MG584844, MG601485 and MG601486 (Table S2).

The effect of *CoERG11* DNA polymorphisms on the predicted protein was evaluated with three bioinformatics tools: SIFT (Sorting Intolerant From Tolerant; http://sift.jcvi.org),[30] PHYRE2 (Protein Homology/analogY Recognition Engine v. 2.0; http://www.sbg.bio.ic.ac.uk/phyre2/html)[31] and PROVEAN (PROtein Variation Effect ANalyser).[32]

Construction of the SAT1 flipper cassette carrying the A395T CoERG11 mutation

To transfer an entire functional copy of the *CoERG11* gene bearing the A395T substitution, the plasmid PSFS2 containing the *SAT1* flipper cassette elements was used as a genetic background to construct the transformation cassette.[33]

A downstream 3′ homology *CoERG11* region (574 bp) was amplified by PCR using DreamTaq Polymerase (Thermo Scientific, Waltham, MA, USA) from the genome of *C. orthopsilosis* ATCC 96139 reference strain, using primers ERG11IFSacII and ERG11ERSacI containing engineered SacII and SacI restriction sites (Table S1). The PCR product was purified using the QIAquick PCR purification Kit (Qiagen, Milan, Italy) according to the manufacturer's guidelines. Next, PSFS2 plasmid DNA and the purified PCR product were both digested with SacI and SacII (New England Biolabs, Ipswich, MA, USA) and ligated using the T4 ligase enzyme (New England Biolabs), generating the P3′HOM.ERG11 plasmid (Table S3), which was propagated in *E. coli DH5α*-competent cells. An upstream 5′ homology fragment (1716 bp) comprising the entire *CoERG11* coding sequence was amplified from a *C. orthopsilosis* fluconazole-resistant clinical isolate (Co681, MIC = 64 mg/L) by PCR using Q5 Taq Polymerase (New England Biolabs) with primers ERG11EFApaI and ERG11ERXhoI containing ApaI- and XhoI-engineered sites (Table S1). Both *CoERG11* and P3′HOM.ERG11 plasmid were digested with ApaI and XhoI enzymes and ligated to generate the plasmid PERG11 (Table S3). Plasmid DNA was then extracted from *E. coli* and amplified using CORT0E05900Rtfwd and CORT0E05900r1 primers (Table S1). The PCR product was purified (Qiagen) for sequencing in order to verify the presence of the A395T mutation (GATC Biotech AG). The PERG11 plasmid was double digested with SacI and ApaI, producing a 6450 bp fragment containing the *SAT1* flipper cassette flanked by the *CoERG11* sequence at the 5′ end and the 3′ homology region. The cassette was gel purified and used in *C. orthopsilosis* transformation experiments.

Transformation of a C. orthopsilosis fluconazole-susceptible strain with the SAT1 flipper construct carrying the A395T CoERG11 mutation

The fluconazole-susceptible *C. orthopsilosis* Co287 (MIC = 2 mg/L) was selected as the parental strain for transformation experiments. *C. orthopsilosis* Co287 strain was transformed by electroporation as previously described[34] with minor modifications. Approximately 4 μg of gel-purified disruption cassette was electroporated in strain Co287 with the following conditions: 2.5 kV, 25 μF and 200 ohms (GenePulser, Bio-Rad, Milan, Italy). Negative and positive transformation controls were also included, to check for nourseothricin susceptibility and cell viability, respectively. Following a 3 h recovery in 100 μL of fresh YPD medium at 30°C, 900 μL of YPD was added and cells were plated on YPD agar supplemented with the selective agent (100 mg/L nourseothricin). Plates were incubated for 48 h at 30°C. Nourseothricin-resistant clones were screened by PCR. Genomic DNA extraction was performed as previously described.[35] Briefly, one colony was suspended in 100 μL of a solution containing (200 mM LiOAc; 1% SDS) incubated at 70°C for 15 min and then precipitated with 96% ethanol. Correct

Table 1. Antifungal susceptibility testing of *C. orthopsilosis* clinical isolates

	MIC EUCAST (mg/L)				Amino acid substitution					
Isolate	POS	VRC	ITC	FLC	V58I	Y132F	Q211K	S420F	A421V	V485I
Rome1	0.03	0.03	0.03	2	V	Y	Q	F	A	V
Rome2	0.03	0.03	0.03	1	V	Y	Q	F	A	V
Rome3	0.03	0.03	0.06	1	V	Y	Q	F	A	V
Rome4	0.06	0.06	0.25	2	I	Y	Q	S	A	V
Rome5	0.06	0.06	0.125	2	I	Y	Q	S	A	V
Rome6	0.06	0.125	0.125	4	V	Y	Q	F	A	V
Rome7	0.25	0.25	0.25	2	I	Y	Q	S	heterozygous	heterozygous
Rome8	0.03	0.125	0.125	2	I	Y	Q	S	A	V
Rome9	0.125	0.25	0.25	4	V	Y	K	S	V	I
Rome10	0.25	0.25	0.25	4	V	Y	K	S	V	I
Rome11	0.25	0.125	0.25	4	V	Y	K	S	V	I
Rome12	0.125	0.25	0.25	4	V	Y	Q	S	V	I
Rome13	0.25	0.25	0.125	4	V	Y	K	S	V	I
Rome14	0.25	0.06	0.125	4	V	Y	K	S	V	I
Rome15	0.125	0.25	0.125	4	V	Y	K	S	V	I
Rome17	0.125	0.125	0.25	4	V	Y	K	S	V	I
Rome19	0.25	0.5	0.25	16	V	Y	K	S	V	I
Rome18	0.25	0.5	0.25	32	V	Y	Q	S	V	I
Rome22	0.5	4	0.5	128	V	F	Q	S	V	I
Rome23	0.125	2	0.25	64	V	F	heterozygous	S	V	I
Rome24	0.125	4	0.125	128	V	F	K	S	V	I
Rome25	0.25	2	0.25	128	V	F	K	S	V	I
Rome26	0.125	4	0.5	128	V	F	heterozygous	S	V	I
Rome27	0.125	4	0.25	64	V	F	K	S	V	I
Rome28	0.25	8	0.25	64	V	F	Q	S	V	I
Rome29	0.125	4	0.25	64	V	Y	Q	S	V	I
Co25	0.125	0.06	0.25	8	V	Y	K	S	V	I
Co47	0.125	0.25	0.25	2	V	Y	K	S	V	I
Co85	0.03	0.125	0.25	4	V	Y	Q	S	A	V
Co124	0.06	0.06	0.5	8	V	Y	Q	F	A	V
Co242	0.125	0.06	0.25	8	V	Y	Q	S	A	V
Co268	0.125	0.06	0.25	16	V	Y	Q	F	A	V
Co269	0.125	0.125	0.125	4	V	Y	Q	F	A	V
Co287	0.03	0.125	0.125	2	V	Y	Q	S	A	V
Co289	0.125	0.125	0.5	4	V	F	Q	S	V	I
Co296	0.125	0.5	0.5	4	V	Y	Q	F	A	V
Co331	0.06	0.5	0.25	4	V	Y	K	S	V	I
Co681	0.125	2	0.5	64	V	F	K	S	V	I
Co687	0.25	2	0.25	64	V	F	K	S	V	I
Co690	0.03	0.5	0.25	1	V	Y	K	S	V	I

POS, posaconazole (R, >0.06 mg/L); VRC, voriconazole (R, >0.25 mg/L); ITC, itraconazole (R, >0.125 mg/L); FLC, fluconazole (R, >4 mg/L).

integration of the cassette was verified using primers Sat1F and OutERG11R (Table S1). Clones that had correctly integrated the cassette were then grown on YNB supplemented with maltose to allow excision of the cassette from the fungal genome. Correct excision was verified by PCR using primers CORT0E05900f1 and CORT0E05900f3 (Table S1). Heterozygosity of the obtained clones was verified by PCR using the primers ERG113HOMF and OutERG11R (Table S1). The presence of the A395T mutation was verified in clones NP1–4 by sequencing an internal fragment of *CoERG11* gene, using PCR and sequencing primers CORT0E05900Rtfwd and CORT0E05900r1 (Table S1).

Phenotypic assays on C. orthopsilosis mutants

C. orthopsilosis Co287-derived mutants and the parental Co287 WT strain were tested for their growth ability in basal conditions. For each clone, a single colony was grown overnight at 37°C in 10 mL of YPD broth, all the strains were diluted to the same OD and were then subcultured in 50 mL of YPD at 37°C under agitation for 24 h. Spectrophotometric readings were taken every 2 h for the first 6 h and then every hour up to 24 h. Antifungal susceptibility testing of the mutant strains was performed as described above.

Results

C. orthopsilosis azole susceptibility testing and identification of CoErg11 amino acid variants

Analysis of susceptibility data obtained from the 40 *C. orthopsilosis* clinical isolates included in the study indicated that 16 isolates were resistant to fluconazole and to at least one other azole antifungal (Table 1). We investigated the sequence variation of the *CoERG11* gene in all fluconazole-resistant and fluconazole-susceptible *C. orthopsilosis* isolates. By comparison of the obtained sequences with that of the reference *C. orthopsilosis* isolate (90–125),[29] 42 different SNPs (Table S4) in 40 isolates were identified, six of which resulted in non-synonymous amino acid substitutions in the corresponding protein (Table 1). Among these, the A395T (amino acid substitution Y132F) was present in 10 of the azole-resistant isolates but in none of the susceptible ones. The other five SNPs were T173G (amino acid substitution V58I), C631A (amino acid substitution Q211K), C1259T (amino acid substitution S420F), C1262T (amino acid substitution A421V) and G1450A (amino acid substitution V485I). Notably, these mutations were present in both susceptible and resistant isolates (Table 1).

In silico analysis of CoErg11 variants

The impact of the six non-synonymous mutations identified in this study on *C. orthopsilosis* Erg11 protein function was predicted using the SIFT, PROVEAN and Phyre algorithms. The SIFT and PROVEAN algorithms predicted the mutation Y132F to be deleterious, potentially affecting protein functionality (score 0.02 and −3.761, respectively) and although analysis with the Phyre suite found a low conservation of the amino acid in position 132, it also predicted a medium level of mutation sensitivity. This program also mapped the substituted amino acid into the active site of the protein (Figure 1). Mutations V58I, Q211K, S420F, A421V and V485I were predicted by the SIFT algorithm to be tolerated; none of them mapped into the protein pocket (data not shown).

Construction of a *C. orthopsilosis* strain carrying a CoERG11 395T mutated allele

To assess the effect of the A395T mutation in the development of azole resistance in *C. orthopsilosis*, the entire *CoERG11* coding sequence from an azole-resistant clinical isolate carrying the mutation was introduced into the *C. orthopsilosis* azole-susceptible isolate Co287, which is WT for the *CoERG11* allele (Table 2), using

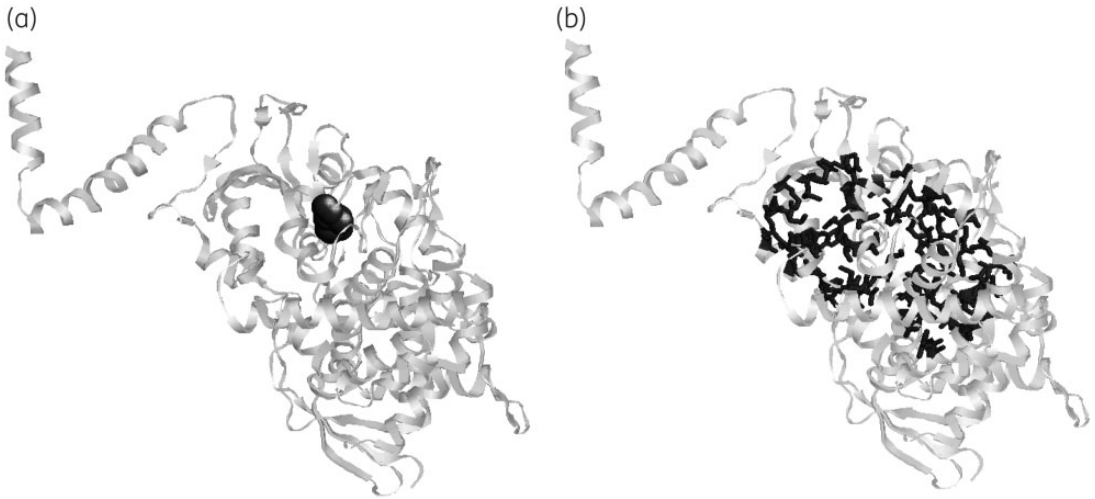

Figure 1. Prediction of PHYRE2 algorithm on the amino acid 132 position in *C. orthopsilosis* Erg11 protein (a) and visualization of the protein pocket (b) according to the *C. albicans* Erg11 resolved structure.[19]

Table 2. Genotyping and azole susceptibility testing of the *C. orthopsilosis* mutant clones obtained in this study

			MIC EUCAST (mg/L)			
Strain	*CoERG11* genotype	Amino acid 132 (CoErg11)	POS	VRC	ITC	FLC
Co287	*ERG11*^{395A}/*ERG11*^{395A}	Y/Y	0.03	0.03	0.25	1
NP1	*ERG11*^{395T}::FRT/*ERG11*^{395A}	Y/F	0.06	1	1	16
NP2	*ERG11*^{395T}::FRT/*ERG11*^{395T}::FRT	F/F	0.06	1	1	32
NP3	*ERG11*^{395T}::FRT/*ERG11*^{395A}	Y/F	0.06	0.25	0.5	16
NP4	*ERG11*^{395A}::FRT/*ERG11*^{395A}::FRT	Y/Y	0.06	0.06	0.125	1

POS, posaconazole; VRC, voriconazole; ITC, itraconazole; FLC, fluconazole.

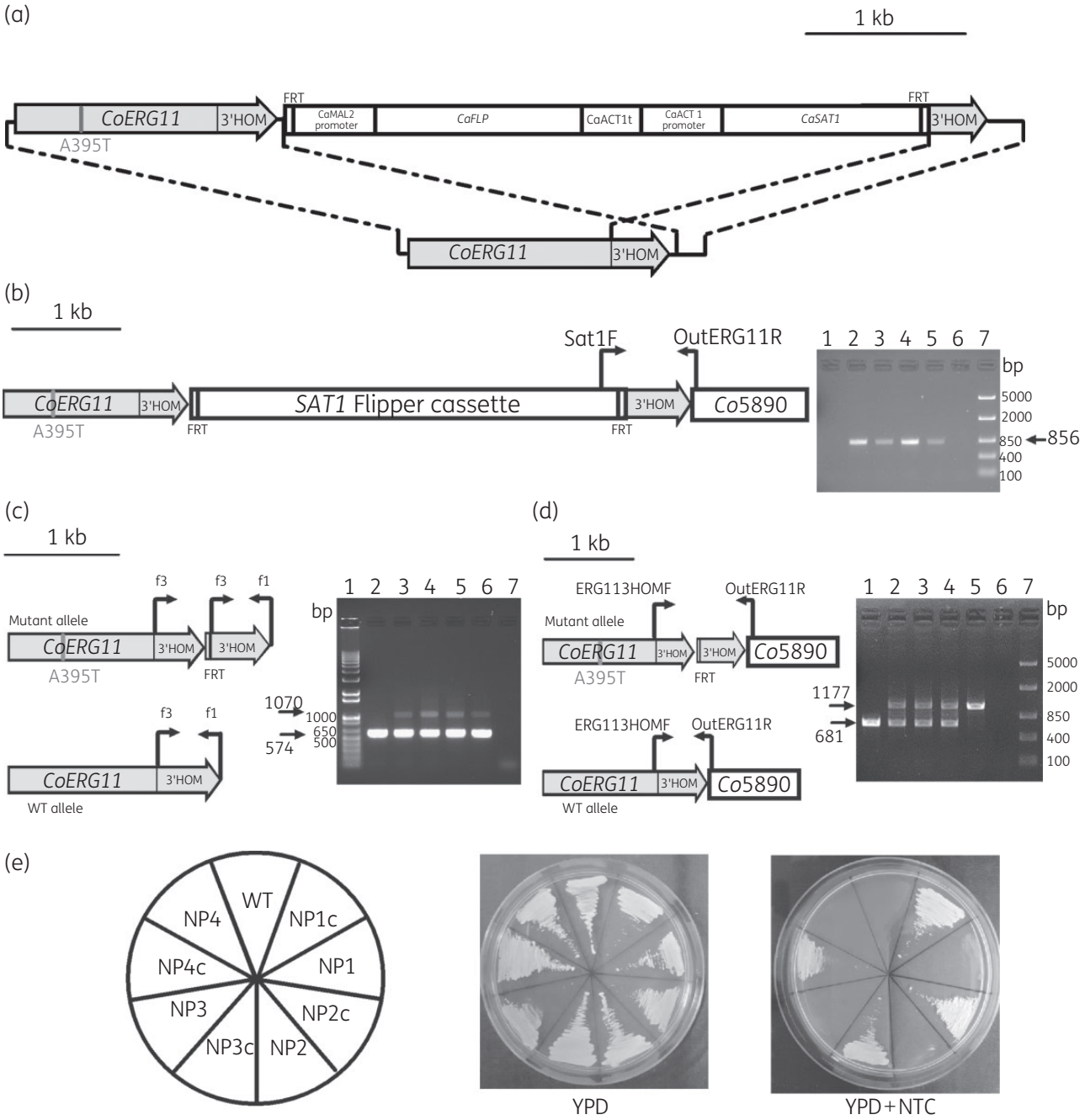

Figure 2. (a) Structure of the *SAT1* flipper cassette used for the integration of *CoERG11* gene carrying the A395T mutation. The cassette relies on a *C. albicans* adapted nourseothricin dominant resistant marker (Ca*SAT1*) under the control of a *C. albicans* actin promoter (CaACT1). Recombinase *FLP* gene, whose expression is driven from the *C. albicans* MAL2 promoter gene (Ca*MAL2*), results in the cassette excision by using flipper recombinase target sequences (FRT). Flanking downstream 3′ homology region contains the 3′ end of the *CoERG11* gene and 217 bp flanking region outside the gene, while upstream 5′ region containing an entire copy of the *CoERG11* containing the A395T mutation. (b) Evaluation of correct integration of *SAT1* flipper cassette in *CoERG11* locus by PCR, using primers Sat1F and OutERG11R. 1, Co287 WT; 2, Co287 NP1; 3, Co287 NP2; 4, Co287 NP3; 5, Co287 NP4; 6, negative control; 7, FastRuler Middle Range DNA Ladder (Thermo Scientific). The expected fragment size in mutant strains was 856 bp. (c) PCR attesting correct excision of *SAT1* flipper cassette. 1, TrackIt™ 1 Kb Plus DNA Ladder (Invitrogen); 2, Co287 WT; 3, Co287 NP1; 4, Co287 NP2; 5, Co287 NP3; 6, Co287 NP4; 7, negative control. Expected fragment sizes: 1070 bp (mutated allele) and 574 bp (WT allele). (d) Discrimination of heterozygous and homozygous strains carrying the A395T mutation. 1, Co287 WT; 2, Co287 NP1; 3, Co287 NP3; 4, Co287 NP4; 5, Co287 NP2; 6, negative control; 7, FastRuler Middle Range DNA Ladder (Thermo Scientific). Expected fragment sizes in heterozygous strains: 1177 and 681 bp. Expected fragment sizes in homozygous mutant strains: 1177 bp and WT strain 681 bp. (e) WT and mutant strains grown on YPD plates and nourseothricin-supplemented YPD plates at 30°C for 24 h. NP1c, NP2c, NP3c and NP4c, mutant strains containing the cassette; NP1, NP2, NP3 and NP4, mutant strains following flippase activation and cassette excision. NTC, nourseothricin.

the *SAT1* flipper cassette strategy (Figure 2a). Following the first round of transformation, four *C. orthopsilosis* Co287-derived clones (NP1–4) integrated the cassette into their genome since they were able to grow under selective conditions (nourseothricin/YPD plates). The correct cassette integration in the *CoERG11 locus* was verified by PCR (Figure 2b) performed on genomic DNA obtained from the NP1–4 clones. Following flippase activation, genomic DNA was extracted and cassette excision was verified by PCR (Figure 2c) and by the absence of growth onto nourseothricin-supplemented YPD plates (Figure 2e).

To verify the presence of the A395T mutation in the *CoERG11* locus, the corresponding region was sequenced in all the clones obtained (Table 2). Three clones (NP1–3) carried the A395T mutation: the NP1 and NP3 clones were heterozygous for the A395T mutation (accession numbers MG543290 and MG543292, respectively), while clone NP2 was homozygous for the A395T mutation (accession number MG543291). PCR analysis further confirmed the replacement of both alleles with the mutated version of the gene in the NP2 clone (Figure 2d). Clone NP4, although correctly integrating the cassette in the *CoERG11* locus, did not carry the mutation (Table 2, accession number MG543293).

Phenotypic characterization of the NP1–4 mutant panel

Antimicrobial susceptibility assay was performed for the panel of the obtained mutant clones (NP1–4) as well as for Co287 WT strain. A resistant phenotype to fluconazole (from 8- to 16-fold increase), itraconazole and voriconazole, was observed in clones NP1–3, while WT strain and clone NP4 did not show any change in susceptibility (Table 2). Interestingly, clone NP2, in which both alleles carried the A395T substitution, showed the highest MIC values of fluconazole (16-fold increase) and voriconazole (>30-fold) among the mutant strains (Table 2). To verify that the increased resistance of mutants did not relate to a direct effect of the mutation on the basal growing ability, the growth of mutants NP1–3 was compared with that of Co287 WT isolate in YPD broth medium at 37°C. No difference was observed in growth rate among the mutants and the WT strains (Figure 3).

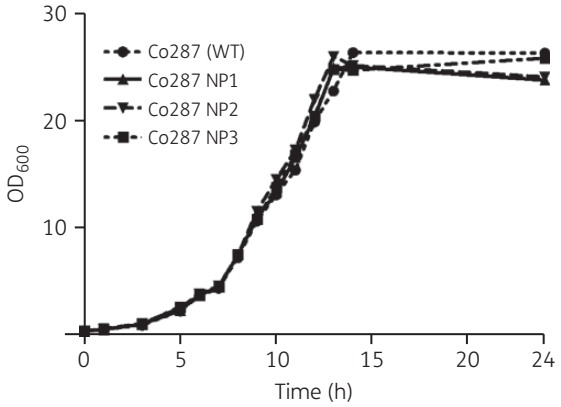

Figure 3. Growth curve of Co287 WT strain and mutant derivative clones Co287 NP1, NP2 and NP3 in YPD liquid medium at 37°C.

Discussion

In the present study, a panel of *C. orthopsilosis* clinical isolates collected from two different geographical regions of Italy was selected and evaluated for azole antifungal susceptibility. A high number of multi-azole-resistant isolates was observed among isolates collected from both regions. This is interesting in view of recent reports indicating that resistant non-*albicans Candida* species are increasingly isolated in the hospital setting, most likely due to the selection of acquired resistance during medical treatments. It has been reported that 20%–30% of candidaemia cases involve species with intrinsic resistance to either fluconazole or echinocandins.[36] To better understand the molecular mechanisms that underlie the azole-resistant phenotype in *C. orthopsilosis*, we sequenced the orthologue of *C. albicans ERG11* (*CORT_0E05900*) in both azole-resistant and azole-susceptible *C. orthopsilosis* clinical isolates. Indeed, mutations in the *ERG11* sequence leading to amino acid substitutions in the encoded lanosterol 14α-demethylase have been demonstrated to contribute to *C. albicans* azole resistance.[12,37–40] Point mutations in *ERG11* orthologues have also been associated with azole resistance in *C. tropicalis*,[41] *C. parapsilosis*[24] and, more recently, in the multiresistant *Candida auris*.[42] However, no data are available on azole resistance in *C. orthopsilosis*. Sequencing results highlighted six mutations linked to non-synonymous amino acid substitutions in our panel of *C. orthopsilosis* clinical isolates, out of which only Y132F was predicted to be damaging and deleterious. This finding is in agreement with what has been previously described for other *Candida* species.[12,24,37–39,41,42] Notably, the mutation was carried by azole-resistant isolates only (fluconazole MIC ranging between 8 and 128 mg/L) and was never observed in azole-susceptible strains. Substitution of tyrosine with phenylalanine could lead to an increase in the hydrophobicity of the active site in Erg11p, resulting in a reduced affinity of the enzyme for fluconazole, which is a hydrophilic molecule.

To demonstrate that Y132F is responsible for azole resistance in *C. orthopsilosis* we used a *SAT1* flipper-driven transformation to replace a WT copy of *CoERG11* with a functional copy of the gene carrying the A395T variant in a fluconazole-susceptible clinical isolate. Upon cassette excision from *C. orthopsilosis* genome, both heterozygous and homozygous clones were obtained, carrying one of either copy of the mutated *CoERG11* allele, respectively. Phenotypic tests were performed on the panel of *CoERG11T* mutant strains to rule out any potential effect of the induced mutation on growth ability in conventional media. Susceptibility testing indicated that *C. orthopsilosis* mutant clones carrying the 395T variant (NP1–3) acquired a resistant phenotype to fluconazole, with the homozygous strain showing the higher increase in fluconazole MIC (from 2 to 32 mg/L). Moreover, the acquisition of one mutated *CoERG11* allele was sufficient to induce an azole-multiresistant phenotype, with MIC values for itraconazole and voriconazole increasing from 0.125 and 0.03 mg/L (classified as susceptible) to 0.25 and 0.25–1 mg/L (classified as resistant), respectively. As expected, the homozygous *CoERG11*–395T mutated strain displayed the highest MIC values for azoles. The introduction of a mutated allele produced a net change in fluconazole susceptibility, from a susceptible to a resistant phenotype, although the mutated strains did not reach the fluconazole MIC value observed for the 'donor strain': this could be explained by the fact that the mutated allele is introduced in a completely different genomic background. Other *C. orthopsilosis* clinical

isolates showed an azole-resistant phenotype in the absence of the Y132F substitution. Azole resistance can originate from different molecular mechanisms, e.g. overexpression of the target, of efflux pumps, or other alterations in sterol biosynthesis, as demonstrated for other *Candida* species, including *C. parapsilosis*.[43] Future studies need to be undertaken to unravel the mechanisms underlying azole resistance in these clinical isolates.

In conclusion, this study presents, to the best of our knowledge, the first assessment of the molecular mechanisms involved in fluconazole resistance in clinical isolates belonging to the emerging species *C. orthopsilosis*, demonstrating that a Y132F substitution in CoErg11p is sufficient to confer multi-azole resistance.

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Transparency declarations

None to declare.

Supplementary data

Tables S1–S4 and Figure S1 are available as Supplementary data at *JAC* Online.

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