

## Molecular Mechanisms of Itraconazole Resistance in *Candida dubliniensis*

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It has previously been shown that overexpression of the *CdMDR1* gene is a major contributor to resistance in fluconazole-resistant isolates of *Candida dubliniensis*. However, since *CdMdr1p* does not mediate transport of other azole drugs such as itraconazole, we investigated the molecular mechanisms of stable resistance to itraconazole obtained in three strains of *C. dubliniensis* (two with nonfunctional *CdCDR1* genes and one with functional *CdCDR1* genes) by serial exposure to this antifungal agent in vitro. Seven derivatives that were able to grow on agar medium containing 64 µg of itraconazole per ml were selected for detailed analysis. These derivatives were resistant to itraconazole, fluconazole, and ketoconazole but were not cross resistant to inhibitors. *CdMDR1* expression was unchanged in the seven resistant derivatives and their parental isolates; however, all seven derivatives exhibited increased levels of *CdERG11* expression, and six of the seven derivatives exhibited increased levels of *CdCDR1* expression compared to the levels of expression by their respective parental isolates. Except for one derivative, the level of rhodamine 6G efflux was decreased in the itraconazole-resistant derivatives compared to the level of efflux in their parental isolates, suggesting altered membrane properties in these derivatives. Analysis of their membrane sterol contents was consistent with a defective sterol C5,6-desaturase enzyme (*CdErg3p*), which was confirmed by the identification of mutations in the alleles (*CdERG3*) encoding this enzyme and their lack of functional complementation in a *Saccharomyces cerevisiae* *erg3* mutant. The results of this study show that the loss of function of *CdErg3p* was the primary mechanism of in vitro-generated itraconazole resistance in six of the seven *C. dubliniensis* derivatives. However, the mechanism(s) of itraconazole resistance in the remaining seventh derivative has yet to be determined.

*Candida dubliniensis* is a recently identified yeast species that is phylogenetically closely related to *Candida albicans* (49) and is frequently misidentified as such (34). Although originally identified in specimens recovered from the oral cavities of human immunodeficiency virus (HIV)-infected individuals in Ireland and Australia, *C. dubliniensis* has since been identified in a wide variety of clinical settings throughout the world (13, 21, 38, 48). In the most comprehensive study to date, by use of the criteria of the National Committee for Clinical Laboratory Standards (fluconazole MICs, ≤4 µg/ml for susceptible, 8 to 32 µg/ml for susceptible dose dependent, and ≥64 µg/ml for resistant), the majority of *C. dubliniensis* isolates were found to be susceptible to fluconazole and other commonly used antifungal drugs, including itraconazole, ketoconazole, and amphotericin B (37). However, fluconazole resistance in clinical isolates and the replacement of susceptible strains by resistant derivatives has been documented (21, 31, 36, 41). Interestingly, comparison of the geometric mean MICs of fluconazole, itraconazole, and ketoconazole for 58 isolates each of *C. albicans* and *C. dubliniensis* showed that while the fluconazole MICs for *C. dubliniensis* isolates were below the breakpoint of 64 µg/ml, they were significantly and consistently higher than those for

the *C. albicans* isolates (34). Furthermore, sequential exposure of fluconazole-susceptible clinical isolates of *C. dubliniensis* to increasing concentrations of fluconazole in agar medium led to the recovery of derivatives expressing stable fluconazole-resistant and susceptible-dose-dependent phenotypes (MIC range, 16 to 64 µg/ml) (29–31). It has been suggested that the ability of *C. dubliniensis* to develop resistance to fluconazole may contribute to its ability to successfully colonize the oral cavities of HIV-infected individuals receiving prolonged fluconazole treatment (31). Furthermore, this may explain the apparent recent emergence of this species in this patient cohort.

While fluconazole is still very often the drug of choice for the treatment of candidosis, the emergence of fluconazole-resistant strains and recurrent infections recalcitrant to therapy has led to a reappraisal of treatment strategies for these infections. The triazole derivative itraconazole has a broader spectrum of activity than fluconazole and has been shown to be effective against both *Candida* and *Aspergillus* species (26). Since itraconazole has been used less extensively than fluconazole, clinical resistance is not yet a major problem.

Azole drugs target an enzyme in *Candida* responsible for the 14α-demethylation of 24-methylenedihydrolanosterol, a step in the ergosterol biosynthetic pathway. This enzyme, known as 14α-lanosterol demethylase, is encoded by the *ERG11* gene. Several mechanisms by which *Candida* species can develop resistance to azole antifungal agents have been described: cells can fail to accumulate these agents; the affinity of the 14α-

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demethylase enzyme for these agents can be altered; the cellular content of Erg11p can be elevated; and other enzymes of the ergosterol biosynthetic pathway, such as the sterol C5,6-desaturase, can be inactivated by mutation. A combination of these mechanisms is likely to occur in many clinical azole-resistant isolates, since resistance to azole antifungal agents in *C. albicans* has been shown to develop in a stepwise process over time (7).

Several studies have demonstrated the importance of the overexpression of multidrug transporters in the development of azole resistance in *Candida* species (25, 27, 43, 45). In *C. albicans*, overexpression of the multidrug transporter Cdr1p is associated with resistance to multiple azole drugs, as well as to other unrelated antifungal drugs and particular inhibitors. In contrast, Mdr1p overexpression is associated with resistance to fluconazole and inhibitors but not to itraconazole and ketoconazole (45, 46). In *C. albicans*, overexpression of Cdr1p is the most frequently reported mechanism of resistance to azole drugs (22, 35). However, in *C. dubliniensis* the mechanisms of resistance differ from those of *C. albicans*. Previous studies have shown that in *C. dubliniensis* the primary mechanism of fluconazole resistance is overexpression of the major facilitator CdMdr1p (30, 54). In this species it has been shown that CdCdr1p is important for mediating reduced susceptibility to itraconazole and ketoconazole but is not essential for fluconazole resistance (29). Moreover, Moran et al. (29) found that 35% of *C. dubliniensis* isolates harbor mutated alleles of *CdCDR1*, which encode a truncated nonfunctional CdCdr1p protein. Interestingly, isolates with nonfunctional CdCdr1p all belong to *C. dubliniensis* genotype 1, a group of very closely related isolates described by Gee et al. (10) that have mainly been recovered from HIV-infected individuals, most of whom have received fluconazole treatment, and that constitute the predominant group of *C. dubliniensis* isolates.

The aims of the present study were (i) to measure the incidence of itraconazole resistance in *C. dubliniensis* clinical isolates, (ii) to determine if resistance to itraconazole could be induced by exposing *C. dubliniensis* isolates to itraconazole *in vitro*, and (iii) to investigate the molecular mechanisms involved in itraconazole resistance in this species. The last aim was deemed to be of particular interest, given the inability of CdMdr1p to transport itraconazole and the high prevalence of nonsense mutations in the *CdCDR1* gene.

#### MATERIALS AND METHODS

**Chemicals, enzymes, radioisotopes, and oligonucleotides.** All chemicals used were of analytical grade or molecular biology grade and were purchased from Sigma-Aldrich Chemical Co. (Tallaght, Dublin, Republic of Ireland), BDH (Poole, United Kingdom), or Roche Diagnostics Ltd. (Lewes, United Kingdom). Enzymes for molecular biology procedures were purchased from Promega Corporation (Madison, Wis.), Roche Diagnostics Ltd., or New England Biolabs Inc. (Beverly, Mass.).

Fluconazole powder was a gift from Pfizer Central Research (Sandwich, United Kingdom), and ketoconazole and itraconazole were both gifts from Janssen Pharmaceuticals (Cork, Republic of Ireland). Amphotericin B was a gift from E. R. Squibb (Swords, Republic of Ireland).

The following drugs were all purchased from Sigma-Aldrich: cerulenin, crystal violet, flucytosine, Geneticin, 4-nitroquinoline-*N*-oxide, cycloheximide, benomyl, fluphenazine, 1,10-phenanthroline, and rhodamine 6G (R6G).

**Strains and media.** The *Escherichia coli* strain XL2-Blue (*recA1 endA1 gyrA96 thi-1 hsdR17 supE44 relA1 lac[F' proAB lacI<sup>q</sup> ZΔ M15 Tn10 (Tet<sup>r</sup>) Amy Cam<sup>r</sup>]*) (Stratagene, La Jolla, Calif.) was used as the host strain for the plasmid pBlue-

script IKS(-) (Stratagene). XL2-Blue and derivatives harboring recombinant plasmids were cultured and maintained as described previously (42).

*Saccharomyces cerevisiae* ATCC 4002667  $\Delta$ erg3 was used as the host for the yeast expression vector pYES (4) and its recombinant derivatives. Strain ATCC 4002667 (*MATa his3-Δ1 leu2Δ0 met15Δ0 ura3Δ0 erg3Δ::kanMX4*) is defective in sterol C5,6-desaturation. The *S. cerevisiae*  $\Delta$ erg3 strain and its transformants harboring *CdERG3* were routinely cultured on yeast nitrogen base (Difco Laboratories, Detroit, Mich.) medium supplemented with 50  $\mu$ g of adenine, histidine, leucine, lysine, uracil, and tryptophan per ml (SC-URA medium) and containing 2% (wt/vol) glucose at 30°C. For heterologous expression of *C. dubliniensis* open reading frames (ORFs) cloned in pYES, *S. cerevisiae*  $\Delta$ erg3 transformants were grown on SC-URA medium containing 2% (wt/vol) galactose instead of glucose.

**Culture conditions for *C. dubliniensis* clinical isolates and *in vitro*-generated derivatives.** The 111 clinical isolates of *C. dubliniensis* tested for their fluconazole susceptibilities in the present study were recovered from 106 separate patients. The 58 clinical isolates of *C. dubliniensis* tested for their itraconazole susceptibilities were recovered from 56 separate patients. All isolates were routinely cultured on potato dextrose agar (PDA; Oxoid, Basingstoke, United Kingdom) medium (pH 5.6) at 37°C. For liquid culture, the isolates were grown in yeast extract-peptone-dextrose (YEPD) broth at 37°C in an orbital incubator (Gallenkamp, Leicester, United Kingdom) at 200 rpm.

**Susceptibility testing procedures.** (i) **BMD Susceptibility testing with fluconazole and itraconazole** was carried out by the broth microdilution (BMD) method of Rodriguez-Tudela and Martinez-Suarez (40). All tests were carried out in duplicate. Results were interpreted according to the following MIC breakpoints for itraconazole: susceptible,  $\leq 0.125$   $\mu$ g/ml; susceptible dose dependent, 0.25 to 0.5  $\mu$ g/ml; resistant,  $\geq 1$   $\mu$ g/ml. For fluconazole the following breakpoints were used: susceptible,  $\leq 4$   $\mu$ g/ml; susceptible dose dependent, 8 to 32  $\mu$ g/ml; resistant,  $\geq 64$   $\mu$ g/ml.

(ii) **Amphotericin B susceptibility testing by E-test.** Determination of susceptibility to amphotericin B by the E-test (AB BIODISK, Solna, Sweden) was carried out according to the instructions of the manufacturer.

(iii) **Plate assays.** The susceptibilities of *C. dubliniensis* and *C. albicans* strains to a range of inhibitors and antifungal drugs (R6G, fluphenazine, hygromycin, Geneticin, and cycloheximide) was tested qualitatively by spotting serial dilutions of yeast cultures on complex YEPD medium agar plates, which permit the easy visualization of growth differences between strains. Each plate contained 25 ml of agar in single-vented 90-mm petri dishes. The *Candida* strains were grown overnight at 30°C with constant shaking in YEPD broth. The cultures were firstly diluted to  $2 \times 10^7$  cells/ml in sterile deionized water. Four serial 1:10 dilutions of this first dilution were made, 4  $\mu$ l of each dilution was spotted onto each plate, and the plates were incubated for 48 h at 30°C.

**Induction of itraconazole resistance *in vitro*.** Seven itraconazole-resistant *in vitro*-generated derivatives of *C. dubliniensis* isolates CD36, CD570, and CD519-8 were included in this study. These were CD36-C, CD36-F, CD570-A, CD570-B, CD570-C, CD570-D, and CD519-A. Isolates CD36 (type strain *C. dubliniensis* NCPF 3949) and CD570 belong to *C. dubliniensis* genotype 1, as described by Gee et al. (10), and harbor the naturally occurring nonfunctional *CdCDR1* gene described by Moran et al. (29), while isolate CD519-8 belongs to *C. dubliniensis* genotype 3 and harbors a functional *CdCDR1* gene. A hundred colonies each of the itraconazole-susceptible *C. dubliniensis* isolates were exposed for 48 to 96 h on 90-mm petri dishes containing 25 ml of Emmons Sabouraud dextrose agar (SDA) medium supplemented with the following increasing concentrations of itraconazole: 0.06, 0.125, 0.25, 0.5, 1, 2, 4, 8, 16, 32, and 64  $\mu$ g/ml. For each of these concentrations, two successive sequential subcultures were carried out by using toothpicks to transfer a portion of each colony.

**DNA isolation and Southern hybridization analysis.** Total genomic DNA of *C. dubliniensis* clinical isolates and derivatives was prepared from cells grown for 18 h in YEPD broth cultures, as described by Gallagher et al. (8).

Restriction endonuclease-digested DNA fragments were separated by electrophoresis and transferred to MagnaGraph nylon membranes (MSI, Westboro, Mass.), as described by Sullivan et al. (48). Hybridization reactions were carried out under high-stringency conditions with DNA probes labeled with [ $\alpha$ -<sup>32</sup>P] dATP (Perkin Elmer, Life Sciences Inc., Boston, Mass.) by random primer labeling in a rotary hybridization oven (Hybaid, Middlesex, United Kingdom), as described by Sullivan et al. (49).

Yeast chromosomes were prepared for electrophoresis in agarose plugs by the method of Vazquez et al. (50) and separated by electrophoresis as described by Sullivan et al. (49).

**PCR amplification and cloning of *C. dubliniensis* DNA sequences.** PCR amplification was performed in 100- $\mu$ l reaction volumes containing 100 pmol each of a forward primer and a reverse primer, 10 mM deoxynucleoside triphosphates

TABLE 1. Nucleotide sequences of primers used to amplify *CdERG11* and *CdERG3*

Primer	Sequence <sup>a</sup> (5'-3')	Restriction site	Nucleotide coordinates <sup>b</sup>	Reference
ERG11F	<u>CGCTCGAGCGCAATATGGCTATTGTTGAAACTGTC</u>	<i>Xho</i> I	1 to 21	44
ERG11R	<u>GCTCTAGAGCTTAAAACATACAAGTTTCTCTTTT</u>	<i>Xba</i> I	1587 to 1564	44
ERG3F1	<u>CGCTCGAGCAACCATCCAGAATATTTG</u>	<i>Xho</i> I	453 to 471	This study
ERG3R1	<u>GCTCTAGAGTGAATTGACCGTAATTG</u>	<i>Xba</i> I	986 to 969	This study
NERG3F	<u>CGCTCGAGCTCCAGTCGATGGATTTTCC</u>	<i>Xho</i> I	771 to 790	This study
NERG3R	<u>GCTCTAGAGCCACCAGTAGATTCATTGATT</u>	<i>Xba</i> I	607 to 587	This study
CAE3F	<u>CGAAGCTTATGGATATCGTACTAGAA</u>	<i>Hind</i> III	1 to 18	This study
CDE3R	<u>GCGGATCCTTGAAATATTGAATGGGGG</u>	<i>Bam</i> HI	1202 to 1183	This study

<sup>a</sup> The restriction endonuclease recognition sites included in the primer sequences are underlined.

<sup>b</sup> The nucleotide coordinates of the *C. dubliniensis* gene (where position +1 corresponds to the first base of the ATG translational start codon). The primer pair ERG11F-ERG11R was derived from the primer sequence used for the *C. albicans* gene by Sanglard et al. (44). The primer pair ERG3F1-ERG3R1 was based on the *C. albicans* ERG3 sequence (GenBank accession no. AF069752).

(2.5 mM each), the high-fidelity enzyme and buffers provided with the Expand PCR System (Roche Diagnostics Ltd.), and 100 ng of *C. dubliniensis* genomic DNA. PCRs were carried out in a DNA thermal cycler (Perkin-Elmer, Norwalk, Conn.).

For the amplification of *CdERG11*, the primer set ERG11F-ERG11R (with *Xho*I and *Xba*I restriction sites included at the respective 5' ends; Table 1), which is a modification of the primer set used by Sanglard et al. (44), was used. Reactions were carried out with 30 cycles of denaturation for 1 min at 94°C, primer annealing for 1 min at 55°C, and extension for 1.5 min at 72°C; this was followed by a final incubation at 72°C for 10 min.

A set of primers (primers ERG3F1 and ERG3R1; Table 1 and Fig. 1) was designed on the basis of the sequence of the *C. albicans* ERG3 gene spanning a 530-bp region which is most conserved among *S. cerevisiae*, *Candida glabrata*, and *C. albicans* ERG3 sequences and was used in an amplification reaction with *C. dubliniensis* strain CD36 template DNA. Reactions were carried out with 30 cycles of denaturation for 1 min at 94°C, primer annealing for 1 min at 55°C, and extension for 30 s at 72°C; this was followed by a final incubation at 72°C for 10 min. The amplicon obtained was radiolabeled and used as a probe in Southern hybridization analysis of *Cl*aI-digested CD36 genomic DNA and was found to hybridize to a single 5.5-kb fragment. A second primer set (NERG3F-NERG3R; Table 1 and Fig. 1) was designed on the basis of the sequence of the 530-bp region mentioned above to amplify its flanking sequences by inverse PCR. *Cl*aI-digested CD36 DNA was ligated to itself, and PCR was carried out on religated DNA template with 30 cycles of denaturation for 1 min at 94°C, primer annealing for 1 min at 55°C, and extension for 5 min at 68°C, followed by a 10-min extension at 68°C.

On the basis of the sequences obtained by inverse PCR, a set of primers (primers CAE3F and CDE3R; Table 1 and Fig. 1) was designed to amplify the complete *CdERG3* ORF. Following restriction enzyme digestion, the amplicons were cloned into pBluescript II KS(-) by conventional methods. These clones were used in sequencing reactions carried out by Lark Inc. (Saffron Walden, United Kingdom). Alignments of the nucleotide and amino acid sequences were carried out by using the CLUSTAL W software program (11). For heterologous expression of *CdERG3* in *S. cerevisiae*, the ORFs cloned in pBluescript were subcloned into the *Xba*I-digested yeast expression vector pYES.

**RNA extraction and Northern analysis.** RNA was extracted from *C. dubliniensis* cultures grown to the midexponential phase (optical density at 600 nm, approximately 0.6) in 50-ml volumes of YEPD broth at 37°C with shaking in an orbital incubator, as described by Moran et al. (30). Extractions were carried out by the glass bead disruption method described by Hube et al. (12). RNA electrophoresis, transfer, and hybridization were carried out as outlined by Moran et al. (30). Membranes were then exposed to BioMax MS film (Eastman Kodak Company, Rochester, N.Y.) for 24 to 72 h. For normalization purposes, all membranes were hybridized with a probe homologous to the *C. dubliniensis* gene encoding translation elongation factor 3 (*CdTEF3*). Hybridization levels were analyzed by scanning densitometry (GelWorks 1D Intermediate; Ultra-Violet Products Ltd., Cambridge, United Kingdom) and normalized with the *CdTEF3* expression levels.

**Measurement of glucose-induced R6G efflux.** Efflux of R6G was determined by a modification of the method of Maesaki et al. (23). Briefly, yeast cells were grown in YEPD broth at 37°C overnight. A total of 10<sup>8</sup> cells were transferred to 100 ml of fresh YEPD broth and incubated at 37°C for 4 to 5 h. The cells were harvested by centrifugation at 3,000 × *g* for 5 min. The pellets were washed twice in phosphate-buffered saline (PBS) and were then resuspended in 10 ml of PBS at a concentration of 10<sup>8</sup> CFU/ml and incubated at 37°C for 1 h in a shaking

incubator. A final concentration of 10 μM R6G was then added to the cell suspension; and the mixture was incubated at 37°C in a shaking incubator for 30 min, after which 1-ml samples were withdrawn and centrifuged at 20,800 × *g* in 1.5-ml microcentrifuge tubes. The supernatants were collected, and the absorption was measured at 527 nm. To observe the effect of glucose, the remainders of the cell suspensions were divided equally and placed in two 50-ml Falcon tubes, one of which was supplemented with 10 μM glucose, while an equal volume of PBS was added to the other. Both suspensions were incubated at 37°C for a further 30 min. One-milliliter samples were withdrawn following incubation and were processed as described above. The concentration of R6G was determined by using a standard curve of R6G concentrations.

**Sterol extraction and analysis. (i) Extraction method for qualitative spectrophotometric analysis.** For qualitative spectrophotometric analysis, total sterols were extracted from cells grown overnight in YEPD broth at 37°C (unless stated otherwise) by a modification of the method described by Arthington-Skaggs et al. (1). Briefly, cells from 1 ml of an overnight broth culture were harvested by centrifugation, and the pellet was resuspended in 0.5 ml of a solution containing 20% (wt/vol) KOH and 60% (vol/vol) ethanol. The suspension was incubated at 95°C for 1 h. Following incubation, 0.6 ml of hexane was added and the samples were vortexed for a few seconds before centrifugation at 20,800 × *g* for 2 min. Following centrifugation, the upper hexane layer containing the sterols was removed and put into fresh tubes. The samples were then diluted by the addition of 0.6 ml of hexane before their absorption between 240 and 330 nm was measured with a spectrophotometer (Genosys 2; ThermoSpectronic, AGB, Dublin, Republic of Ireland).

**(ii) Extraction method for GC-MS.** For gas chromatography-mass spectrometry (GC-MS), nonsaponifiable sterols were extracted by a modification of the method described by Venkateswarlu et al. (51). Single colonies from the plate cultures were inoculated into 15 ml of YEPD broth and incubated overnight at 30°C and 200 rpm. Cells were recovered by centrifugation at 1,500 × *g* for 10 min and washed once in 20 ml of sterile water. Each cell pellet (approximately 400 mg [wet weight]) was resuspended in 3 ml of methanol and transferred to a stoppered glass test tube. Then, 2 ml of 60% (wt/vol) potassium hydroxide and 2 ml of 0.5% (wt/vol) pyrogallol dissolved in methanol were added to each tube, and the tubes were gently vortexed to mix the contents. The tubes were stoppered and placed in a water bath at 90°C for 2 h and then removed and allowed to cool

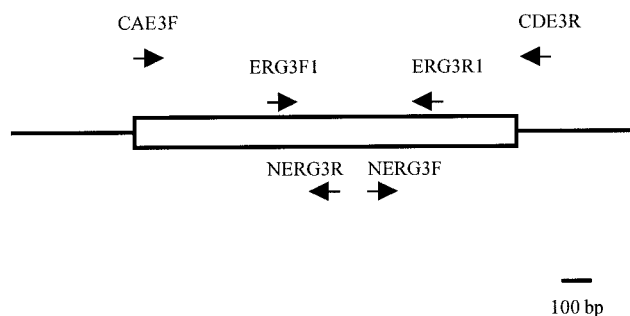


FIG. 1. Map of the *CdERG3* region. The rectangular box represents the *CdERG3* ORF. Arrows indicate the positions of the primer sequences used in this study.

TABLE 2. Susceptibilities of parental *C. dubliniensis* isolates and itraconazole-resistant derivatives to antifungal agents and inhibitors

Strain name	Parent or derivative	MIC ( $\mu\text{g/ml}$ ) <sup>a</sup>											
		ITRA	FLU	KETO	AMB	BEN	CER	CRY	CYH	5FC	NQO	PHE	R6G
CD36	Parental isolate	0.03	0.5	0.03	0.015	32	1	0.06	32	0.125	0.25	2	0.5
CD36-C	CD36 derivative	64	256	2	0.125	32	0.5	0.03	32	0.125	0.06	2	0.25
CD36-F	CD36 derivative	64	256	2	0.125	32	0.5	0.03	32	0.125	0.06	2	0.25
CD570	Parental isolate	0.03	0.5	0.03	0.03	32	1	0.06	32	0.125	0.125	4	0.5
CD570-A	CD570 derivative	64	256	2	0.025	32	1	0.03	32	0.125	0.125	2	0.25
CD570-B	CD570 derivative	64	256	4	0.06	32	0.5	0.03	16	0.125	0.03	2	0.5
CD570-C	CD570 derivative	64	256	2	0.06	16	0.5	0.03	16	0.125	0.03	1	0.25
CD570-D	CD570 derivative	32	256	1	0.06	16	0.5	0.03	32	0.125	0.03	1	0.25
CD519-8	Parental isolate	0.06	0.5	0.03	0.03	64	0.5	0.5	128	0.125	0.06	4	1
CD519-8A	CD519-8 derivative	32	128	4	0.25	64	0.5	0.25	128	0.125	0.03	4	0.5

<sup>a</sup> MICs were determined by the BMD method. Abbreviations: ITRA, itraconazole; FLU, fluconazole; KETO, ketoconazole; AMB, amphotericin B; BEN, benomyl; CER, cerulenin; CRY, crystal violet; CYH, cycloheximide; 5FC, flucytosine; NQO, 4-nitroquinoline-*N*-oxide; PHE, 1,10-phenanthroline; R6G, rhodamine 6G.

to room temperature. Then, 10 ml of hexane was added to each tube, and the tube was vortexed vigorously for 60 s. Following vortexing, the phases were allowed to separate, the upper hexane layer was removed with a glass capillary pipette and placed in a clean glass test tube, and the hexane was evaporated off under nitrogen. Following silylation for 1 h at 60°C with *N,O*-bis(trimethylsilyl) trifluoroacetamide (50  $\mu\text{l}$ ) in 50  $\mu\text{l}$  of toluene, the sterols were separated and identified by GC-MS (VD 12-250; VG Biotech, Manchester, United Kingdom).

**Nucleotide sequence accession number.** The sequence of *CdERG3* has been deposited in the EMBL nucleotide sequence database under accession no. AJ421248.

## RESULTS

***C. dubliniensis* fluconazole and itraconazole susceptibility testing.** The susceptibilities of *C. dubliniensis* clinical isolates were determined in an attempt to determine the levels of itraconazole and fluconazole resistance. The fluconazole susceptibilities of 111 *C. dubliniensis* isolates from 106 separate patients were assessed by BMD. The vast majority of the isolates (105 of 111; 94.6%) exhibited fluconazole-susceptible phenotypes (MIC range, 0.125 to 4  $\mu\text{g/ml}$ ) when tested by BMD. The itraconazole susceptibilities of 58 of the 111 *C. dubliniensis* isolates from 56 separate patients were also assessed by BMD. The vast majority of these isolates (52 of 58; 89.6%) yielded itraconazole-susceptible phenotypes (MIC range, 0.03 to 0.125  $\mu\text{g/ml}$ ). Six isolates were susceptible dose dependent to itraconazole (MIC range, 0.25 to 0.5  $\mu\text{g/ml}$ ), but none of the 58 isolates tested were found to be resistant to itraconazole (MIC, >1  $\mu\text{g/ml}$ ).

**Generation of itraconazole-resistant derivatives by in vitro exposure.** Previously, it was shown that stable fluconazole resistance in *C. dubliniensis* could be induced in vitro by serial subculture on agar plates containing increasing concentrations of drug (31). In order to determine whether stable itraconazole resistance could be induced in *C. dubliniensis* by exposure to the drug and to investigate the genetic basis of itraconazole resistance in *C. dubliniensis*, 100 colonies each of three separate itraconazole-susceptible *C. dubliniensis* isolates (CD36, CD570, and CD519-8) were exposed to increasing concentrations of itraconazole by subculture on Emmons SDA containing itraconazole. *C. dubliniensis* isolates CD36 and CD570 were chosen because they harbored a naturally occurring non-functional version of the *CdCDR1* gene due to the presence of a nonsense mutation described by Moran et al. (29), while the

third isolate, CD519-8, was chosen because it harbored a functional *CdCDR1* gene. Isolates CD36 and CD570 belong to *C. dubliniensis* genotype 1, while isolate CD519-8 belongs to genotype 3 (10). All colonies grew well on initial culture on Emmons SDA containing 0.06  $\mu\text{g}$  of itraconazole per ml. Following an additional subculture on Emmons SDA containing 0.06  $\mu\text{g}$  of itraconazole per ml and successive sequential subcultures on Emmons SDA containing increasing concentrations of itraconazole (0.125, 0.25, 0.5, 1, 2, 4, 8, 16, 32, and 64  $\mu\text{g/ml}$ ), 4 derivatives of CD36, 10 derivatives of CD570, and 4 derivatives of CD519-8 that could grow on agar containing 64  $\mu\text{g}$  of itraconazole per ml were recovered. After subculture on drug-free PDA medium, all derivatives were assessed for their susceptibilities to itraconazole and fluconazole by BMD. The itraconazole and fluconazole MICs for 2 of the 4 CD36 derivatives (CD36-C and CD36-F), 4 of the 10 CD570 derivatives (CD570-A, CD570-B, CD570-C, and CD570-D), and 1 of the 4 CD519-8 derivatives (CD519-8A) were significantly higher than those for their respective parental isolates (Table 2). In addition, the ketoconazole and amphotericin B MICs for all seven derivatives were higher than those for their respective parental isolates (Table 2). The stabilities of the itraconazole and fluconazole resistance phenotypes of these specific derivatives were assessed by monitoring the MICs for the strains following 15 successive subcultures on drug-free PDA and following prolonged storage and subculture over a period of 6 to 36 months. For each of the seven derivatives examined, no change in MIC was detected by BMD following subculture or storage. The remaining derivatives, which did not show stable resistance to itraconazole, were considered to exhibit transient resistance to the drug.

Because resistance to azole drugs is often associated with resistance to inhibitors, additional susceptibility tests with a range of drugs and inhibitors were performed following subculture on drug-free PDA and following prolonged storage to further characterize the phenotypes of the derivatives. However, the MICs of the inhibitors cycloheximide, benomyl, flucytosine, 1,10-phenanthroline, 4-nitroquinoline-*N*-oxide, cerulenin, crystal violet, and R6G for the derivatives were found to be either the same or two to fourfold lower than the corresponding MICs for their respective parental isolates (Table 2).

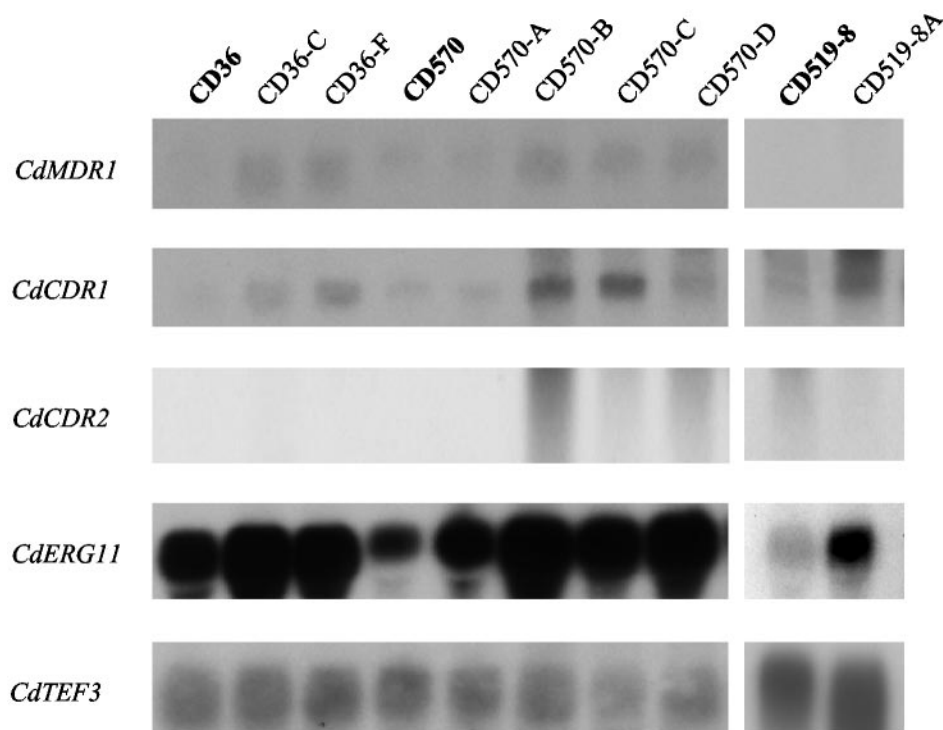


FIG. 2. Northern blots of total RNA from azole-resistant derivatives CD36-C, CD36-F, CD570-A, CD570-B, CD570-C, CD570-D, and CD519-8A and their azole-susceptible parental isolates, CD36, CD570, and CD519-8. Total RNA was extracted from exponentially growing cultures, and 15  $\mu$ g was electrophoresed on a denaturing agarose gel. Following transfer to a nylon membrane, the blots were sequentially probed with radiolabeled DNA probes homologous to *CdMDR1*, *CdCDR1*, *CdCDR2*, and *CdERG11* and the constitutively expressed *CdTEF3* gene. Expression of *CdTEF3* was used as an internal control for RNA loading.

**Phenotypic and molecular analyses of itraconazole-resistant derivatives.** In order to ensure that the derivatives were clonal with their respective parental isolates and not exogenous itraconazole-resistant contaminants, the derivatives and their parental isolates were thoroughly examined by a variety of phenotypic and molecular tests. When cultured on itraconazole-free PDA, the in vitro-generated itraconazole-resistant derivatives exhibited the same smooth, white colony morphology as their parental isolates. All the derivatives yielded *C. dubliniensis*-specific substrate assimilation profiles with the API ID32C yeast identification system, and all yielded *C. dubliniensis*-specific amplimers when tested by PCR with the *C. dubliniensis*-specific primers described by Donnelly et al. (3) (data not shown). Hybridization analysis of *EcoRI*-digested total cellular DNA from strains CD36, CD570, and CD519-8 and their respective derivatives was carried out with *C. dubliniensis*-specific fingerprinting probe Cd25, which can be used to discriminate between *C. dubliniensis* isolates (10, 14). Each of the three itraconazole-susceptible parental isolates, CD36, CD570, and CD519-8, yielded distinct Cd25-generated fingerprint profiles (data not shown). The itraconazole-resistant derivatives of CD36 (CD36-C and CD36-F), CD570 (CD570-C and CD570-D), and CD519-8 (CD519-8A) yielded fingerprint profiles identical to those of their respective parental isolates. Minor band variations in the hybridization patterns of both derivative CD570-A and derivative CD570-B compared to that of their parental isolate, CD570, were detected, suggesting that these derivatives had undergone microevolution in vitro (data

not shown). Karyotype analysis by pulsed-field gel electrophoresis confirmed the relatedness between the derivatives and their respective parental isolates (data not shown). All of these results indicated that the itraconazole-resistant derivatives were clonally related to their respective parental isolates.

***CdERG11* expression in azole-resistant derivatives.** In order to determine if changes in the expression of *CdERG11* were involved in the azole resistance phenotype observed in the seven azole-resistant derivatives of the present study, the mRNA levels of the *CdERG11* gene were analyzed by Northern blot analysis. *CdERG11* mRNA was detected in the three parental strains, CD36, CD570, and CD519-8, and their azole-resistant derivatives, CD36-C, CD36-F, CD570-A, CD570-B, CD570-C, CD570-D, and CD519-8A. However, *CdERG11* was expressed four to eight times as much in all seven azole-resistant derivatives as in their respective parental isolates (Fig. 2).

***CdERG11* sequence analysis of azole-resistant *C. dubliniensis* derivatives.** Mutations affecting the affinity of Erg11p for azole antifungal drugs that render yeast cells resistant to these drugs have been well documented as a drug resistance mechanism (5, 17, 20, 24, 44). The possibility that such a mechanism contributed to the azole resistance phenotype in some or all of the azole-resistant *C. dubliniensis* derivatives was therefore investigated. *CdERG11* genes from *C. dubliniensis* isolates CD36, CD570, and CD519-8 and their derivatives, CD36-C, CD36-F, CD570-A, CD570-B, CD570-C, CD570-D, and CD519-8A, were isolated by PCR with primer pair ERG11F-ERG11R (Table 1) and genomic DNA as the template. In each case, frag-

ments of the expected length (1.6 kb) were obtained. Both strands of the coding region of the *CdERG11* genes from each derivative were sequenced and compared to the corresponding sequences from their respective parental isolates. To reduce PCR error, a proofreading polymerase (Expand PCR system; Roche Diagnostics Ltd.) was used in each case to obtain amplicons. Furthermore, amplicons from five independent PCRs with template DNA from each parental isolate and derivative were sequenced.

There was no amino acid residue difference between the deduced amino acid sequence of the *CdERG11* gene from parental isolate CD36 and the sequence recently published by Perea et al. (36) (GenBank accession no. AY034876). However, there were two heterozygous polymorphisms (R210I and D225Y) in the *CdErg11p* sequence from parental isolate CD570, and there was one heterozygous polymorphism (V402G) and two homozygous polymorphisms (I188V and R499K) in the amino acid sequence from parental isolate CD519-8. Since strains CD36, CD570, and CD519-8 are susceptible to itraconazole and other azole drugs, the sequence differences found in these strains obviously do not contribute to azole resistance. In each case, the *CdERG11* sequences of the itraconazole-resistant derivatives were identical to the corresponding sequences of their respective parental isolates. Therefore, mutation of the *CdERG11* gene could not have been responsible for the resistance phenotype observed in the derivatives.

**Expression of multidrug resistance genes.** Since overexpression of genes encoding efflux pumps have been implicated in azole resistance in *C. albicans* and *C. dubliniensis*, we investigated if the azole resistance of the in vitro-generated derivatives of *C. dubliniensis* was associated with increased levels of expression of the *CdCDR1*, *CdCDR2*, or *CdMDR1* gene using Northern blot analysis. For analysis of *CdCDR1* and *CdCDR2* expression, the cloned 5' ends of these genes were used as probes, as described by Moran et al. (30). For analysis of *CdMDR1* expression, a PCR amplicon spanning the entire ORF was used as a probe. None of the itraconazole-resistant derivatives exhibited significantly elevated levels of *CdMDR1* mRNA compared to the levels for their respective azole-susceptible parental isolates (Fig. 2). However, elevated levels of *CdCDR1* expression were detected in six of the seven azole-resistant derivatives (Fig. 2). There was no significant elevation in the level of *CdCDR1* expression in azole-resistant derivative CD570-A. Large increases in the levels of *CdCDR1* expression were observed in derivatives CD570-B (9-fold) and CD570-C (12-fold). Smaller increases were observed in derivatives CD570-D (4-fold), CD36-C (3-fold), CD36-F (5-fold), and CD519-A (2.5-fold). There was no significant elevation of *CdCDR2* mRNA levels in any of the azole-resistant derivatives (Fig. 2).

**Uptake and glucose-mediated efflux of R6G.** To further investigate the role of energy-dependent efflux mechanisms in the azole-resistant derivatives, the uptake and glucose-mediated efflux of R6G were measured. R6G is a fluorescent compound known to be a substrate of ABC transporters encoded by genes such as *CDR1*. The method used in this study, as described by Maesaki et al. (23), directly assessed the efflux of R6G from energy-starved cells by measuring the extracellular concentrations of R6G following the addition of glucose. In the absence of glucose, the extracellular concentrations of R6G were roughly similar for the azole-susceptible parental isolates

and their respective azole-resistant derivatives (data not shown). In contrast, in the presence of glucose there was an increase in the level of R6G efflux by azole-susceptible parental isolate CD36 (which harbors a defective *CdCDR1* gene) compared to those by its azole-resistant derivatives, CD36-C and CD36-F (Fig. 3). Likewise, a notably increased level of R6G efflux was observed for parental isolate CD519-8 (which harbors a functional *CdCDR1* gene) compared with that observed for its derivative, CD519-8A. This was also the case for azole-susceptible parental isolate CD570, which showed an increased level of R6G efflux compared with those for three of its four azole-resistant derivatives, CD570-B, CD570-C, and CD570-D. However, extracellular R6G levels for azole-resistant derivative CD570-A in the presence of glucose did not show a significant difference compared to those for its azole-susceptible parental isolate, CD570, suggesting no difference in glucose-mediated drug efflux between the parental isolate and its derivative. These results suggest that R6G efflux from the cells mediated by ABC drug transporter extrusion pumps like *CDR1* or *CDR2* was more efficient in the azole-susceptible parental isolates than in six of their seven azole-resistant derivatives and is therefore unlikely to be involved in resistance. A similar reduction of active transport efficiency has previously been associated with changes in the membranes of *erg* mutants of *S. cerevisiae* (15, 32, 47). This led us to investigate whether *erg* mutations may be present in the itraconazole-resistant derivatives described here, as resistance to azole drugs in *Candida* species can be caused by alterations in the ergosterol biosynthesis pathway. In particular, alterations resulting from mutations in the *ERG3* gene encoding the enzyme sterol C5,6-desaturase have been described in *C. albicans* and *S. cerevisiae* (16, 18, 19, 28). In the present study, for comparative purposes, R6G efflux was measured in a *C. albicans* strain with an altered ergosterol pathway (DSY1751, an *erg3* disruptant) (D. Sanglard, unpublished data) and in a wild-type *C. albicans* strain (CAF 2-1) (6). In glucose-starved cells, the extracellular concentration of R6G was similar for the two strains. However, in the presence of glucose there was a marked increase in the level of R6G efflux in the wild-type strain compared to that in the  $\Delta erg3$  strain (Fig. 3). The latter finding was similar to the findings for six of the seven *C. dubliniensis* derivatives, as described above, and suggested that these derivatives could have altered membrane properties.

**Growth in the presence of inhibitors.** It has previously been shown that yeast cells with altered membrane properties are more susceptible to certain inhibitors. Therefore, the azole-resistant derivatives described above and their respective parental isolates were tested on agar medium for their susceptibilities to a range of inhibitors. *C. albicans*  $\Delta erg3$  strain DSY1751 and wild-type *C. albicans* strain CAF2-1 were also tested for comparison. All the derivatives except CD570-A showed increased susceptibilities to fluphenazine, cycloheximide, and Geneticin (Fig. 4). All the derivatives also exhibited increased susceptibilities to R6G. *C. albicans*  $\Delta erg3$  strain DSY1751 also showed increased susceptibilities to these inhibitors compared to those of wild-type *C. albicans* strain CAF2-1. All of these results strongly suggest that the azole-resistant *C. dubliniensis* derivatives have altered membrane properties that result in increased susceptibilities to inhibitors that make their

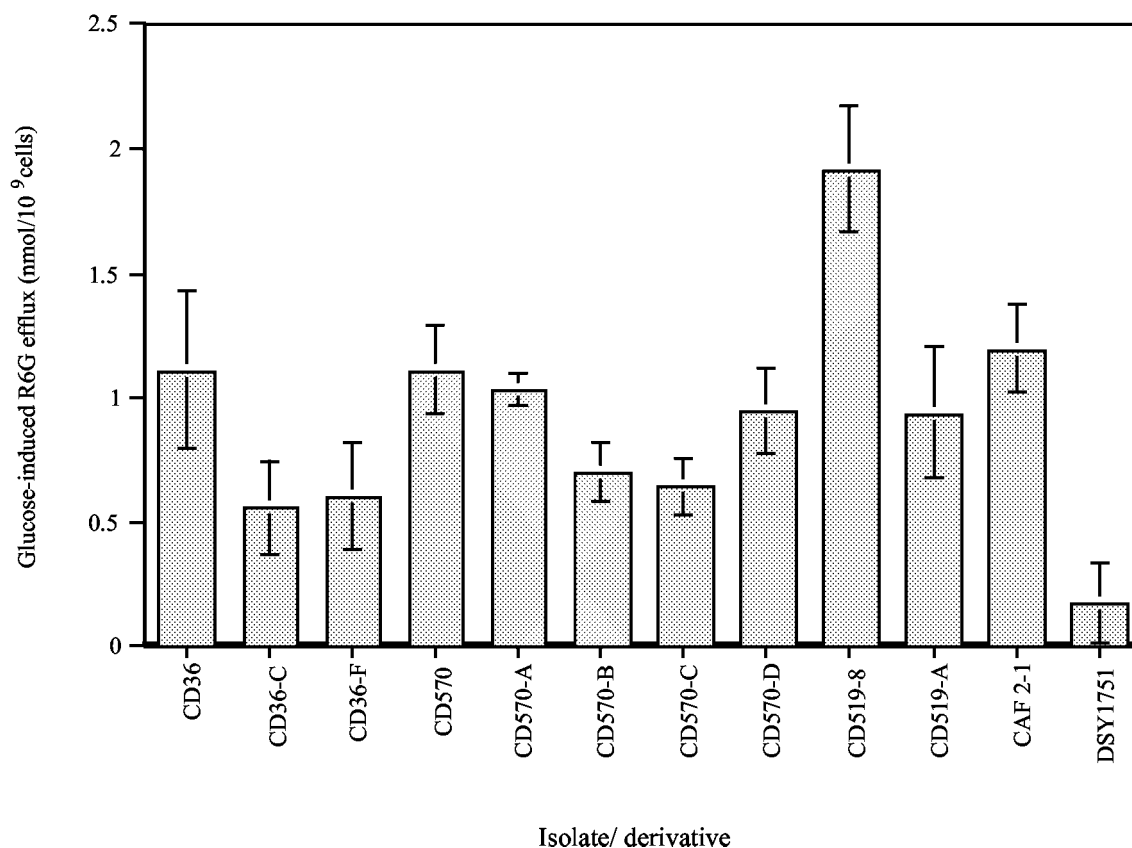


FIG. 3. Glucose-induced R6G efflux from *C. dubliniensis* azole-resistant derivatives CD36-C, CD36-F, CD570-A, CD570-B, CD570-C, CD570-D, and CD519-8A and the azole-susceptible parental isolates CD36, CD570, and CD519-8. For comparative purposes, R6G efflux data for wild-type (CAF2-1) and  $\Delta erg3$  (DSY1751) *C. albicans* strains were included. Cells were incubated with 10  $\mu$ M R6G at 37°C. After 30 min of incubation in glucose-free PBS, glucose was added to a final concentration of 10  $\mu$ M. After a further 30 min of incubation, with or without glucose, at 37°C, the cells were removed by centrifugation and the absorption of the resulting supernatant was measured at 527 nm. The concentration of R6G was calculated by using a standard curve of the R6G concentration. R6G efflux was calculated by subtracting the extracellular R6G concentration in the absence of glucose from the extracellular R6G concentration in the presence of glucose. Each bar indicates the standard error of the mean of at least two sets of experiments.

phenotypes comparable to the phenotype observed for *C. albicans* DSY1751 due to the inactivation of the *ERG3* gene.

**Analysis of sterol contents.** To investigate whether the sterol contents of the azole-resistant *C. dubliniensis* derivatives were different from those of their respective parental isolates and to determine whether alterations in the ergosterol biosynthetic pathway were responsible for azole resistance, the nonsaponifiable sterol contents of the derivatives and their parental isolates were extracted and examined by UV spectrophotometry. The UV absorption spectra of the wild-type sterol preparations from parental isolates CD36, CD570, and CD519-8 had peaks at 261, 273, 282, and 294 nm, which are typical of ergosterol (Fig. 5). These peaks were absent in the spectra of azole-resistant derivatives CD36-C and CD36-F (Fig. 5A); CD570-B, CD570-C, and CD570-D (Fig. 5B); and CD519-8A (Fig. 5C). The absence of the peaks indicated the absence of ergosterol in these derivatives. However, the UV absorption spectrum of azole-resistant derivative CD570-A exhibited the peaks characteristic of ergosterol (Fig. 5B). The UV absorption spectra of *C. albicans*  $\Delta erg3$  strain DSY1751 and wild-type strain CAF2-1 were also examined for comparison. As expected, the spectrum of CAF2-1 exhibited the peaks characteristic of ergosterol,

while the spectrum of DSY1751 sterols lacked these peaks (Fig. 5D) and was reminiscent of the profiles of the *C. dubliniensis* derivatives described above.

The sterols present in all seven derivatives were further analyzed by GC-MS, which allowed their separation and identification. The results of the GC-MS analysis are presented in Table 3. As expected, all of the derivatives except CD570-A lacked ergosterol and mainly accumulated ergosta-7,22-dienol, while the parental isolates accumulated ergosterol (Table 3). Although derivative CD570-A accumulated ergosterol, it accumulated noticeably less ergosta-tetraenol and more unknown sterols than its parental isolate, CD570 (Table 3).

**Cloning and sequencing of the *C. dubliniensis* *ERG3* gene.** Since six of seven azole-resistant derivatives investigated had altered membrane properties, lacked ergosterol, and accumulated mainly ergosta-7,22-dienol (a characteristic for defects in *ERG3*), we hypothesized that they harbored a mutated sterol C5,6-desaturase (*Erg3p*). The sterol C5,6-desaturase is an enzyme involved in ergosterol biosynthesis that is thought to be located in the endoplasmic reticulum, where it catalyzes the introduction of a double bond between C-5 and C-6 in the B ring of the developing sterol molecule. In order to test this

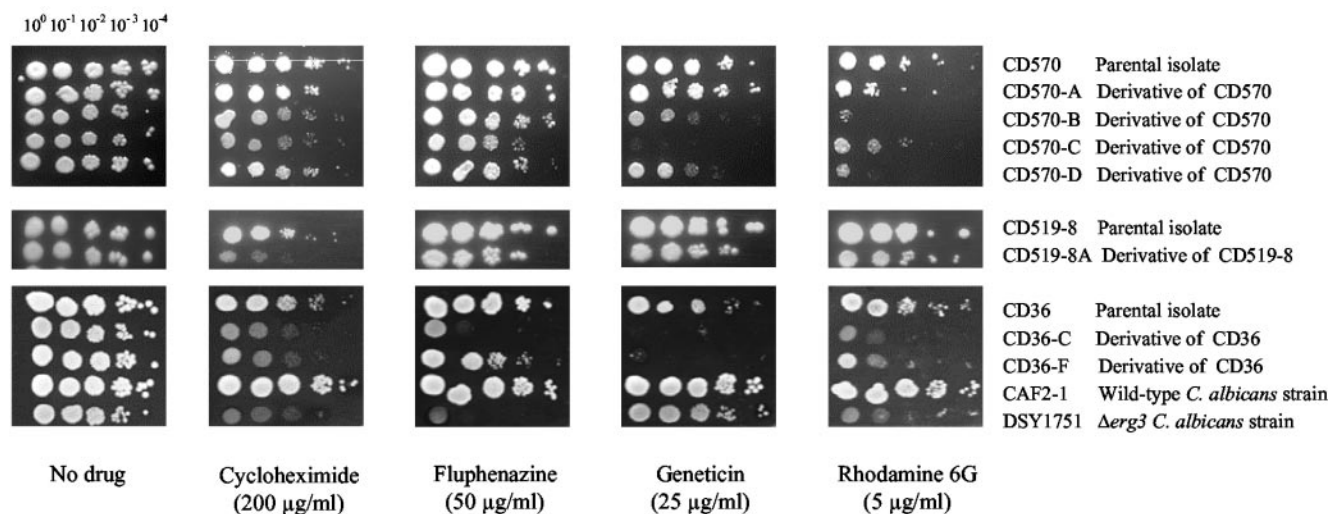


FIG. 4. Susceptibilities of azole-resistant derivatives CD570-A, CD570-B, CD570-C, CD570-D, CD519-8A, CD36-C, and CD36-F and their azole-susceptible parental isolates, CD570, CD519-8, and CD36, to inhibitors. A wild-type *C. albicans* strain (CAF2-1) and a *C. albicans*  $\Delta$ erg3 strain (DSY1751) were included for comparison of phenotypes. Each strain was grown to the exponential growth phase to a density of  $2 \times 10^7$  cells/ml, and 4  $\mu$ l was spotted in a dilution series on YEPD agar plates containing fixed concentrations of inhibitors, as indicated. The plates were incubated for 48 h at 30°C.

hypothesis, it was necessary to clone and sequence the gene encoding sterol C5,6-desaturase in *C. dubliniensis*. Initially, a pair of oligonucleotide primers complementary to the *C. albicans* *ERG3* gene sequence, termed ERG3F1 and ERG3R1 (Table 1; Fig. 1), and a proofreading polymerase (Expand PCR system; Roche Diagnostics Ltd.) were used to amplify a 530-bp internal region of the gene with *C. dubliniensis* CD36 genomic DNA as the template. The 530-bp amplified product obtained was sequenced, and a homology search was carried out with the BLAST computer program. The homology search indicated that the *C. dubliniensis* sequence is highly homologous to the *C. albicans* *ERG3* gene. In order to clone the remainder of the *ERG3* gene, an inverse PCR strategy with the primer pair NERG3F-NERG3R was used (Table 1; Fig. 1). This yielded a fragment of 5.5 kb, both strands of which were sequenced. The reconstructed sequence contained one significant ORF of 1,161 bp. This ORF, termed *CdERG3*, has the capacity to encode a protein of 387 amino acids with a predicted molecular mass of 45.6 kDa and a  $pI$  of 6.61. The *CdERG3* sequence has been deposited in the EMBL nucleotide sequence database (accession no. AJ421248). Alignment of the *CdERG3* gene and the *C. albicans* *ERG3* gene by use of the CLUSTAL W sequence alignment computer program demonstrated that the genes are highly similar, being 91% identical at the nucleotide sequence level (data not shown). The predicted amino acid sequence of CdErg3p shows a high degree of amino acid identity (93.3%) to that of CaErg3p (Fig. 6). The predicted CdErg3p contains four histidine-rich motifs (HX3H, HX2HH, HX2H, and HX2HH), three of which are conserved in all known sequences of sterol C5,6-desaturases (Fig. 6). These histidine box domains are thought to contain the active site of the enzyme and are putative iron-binding domains in *C. albicans* (28).

**Sequencing of *CdERG3* genes of azole-resistant derivatives.** The primer pair CAE3F-CDE3R (Table 1; Fig. 1) was used to amplify the *CdERG3* genes from the azole-susceptible parents

and their azole-resistant derivatives by using a proofreading polymerase (Expand PCR system; Roche Diagnostics Ltd.). In each case, an amplicon of 1.1 kb was obtained. All amplicons were cloned into pBluescript and sequenced. There were no differences between the deduced amino acid sequence of parental isolate CD570 and the corresponding amino acid sequence of CD36. There were two amino acid residue differences (Q47H and V119I) between the deduced amino acid sequence obtained from parental isolate CD519-8 and the sequence obtained from *C. dubliniensis* type strain CD36 described above. Analysis of the sequences obtained for the azole-resistant derivatives revealed that all seven azole-resistant derivatives had alterations in the *CdERG3* gene which conferred amino acid changes. Point mutations were found in derivatives CD36-C, CD36-F, CD570-A, CD570-C, CD570-D, and CD519-8A. Derivatives CD570-B and CD519-8A were found to harbor deletions of 2 bp which would introduce frame shifts during translation and therefore result in the synthesis of truncated proteins (Fig. 6).

Derivative CD36-C harbored two mutations, one of which (H318N) is a substitution of a histidine residue in one of the conserved histidine-rich domains which have been shown to be essential for the desaturase activity of the sterol C5,6-desaturase enzyme (39). The point mutations P272L (in CD36-C), Q160K (in CD36-F), H269N (in CD570-A), and Q327K (in CD570-C and CD570-D) affect amino acid residues conserved in fungal and mammalian sterol C5,6-desaturases (data not shown). As well as the frameshift mutation, derivative CD519-8A harbored four heterozygous point mutations in the N-terminal region of CdErg3p, the least conserved region of the protein (Fig. 6). While two independent derivatives of CD570, CD570-C and CD570-D, shared the same Q327K mutation, all other mutations identified were specific to each derivative. In order to determine whether the mutations identified in the derivatives were homozygous or heterozygous or whether they may have been artifacts of PCR amplification, five indepen-



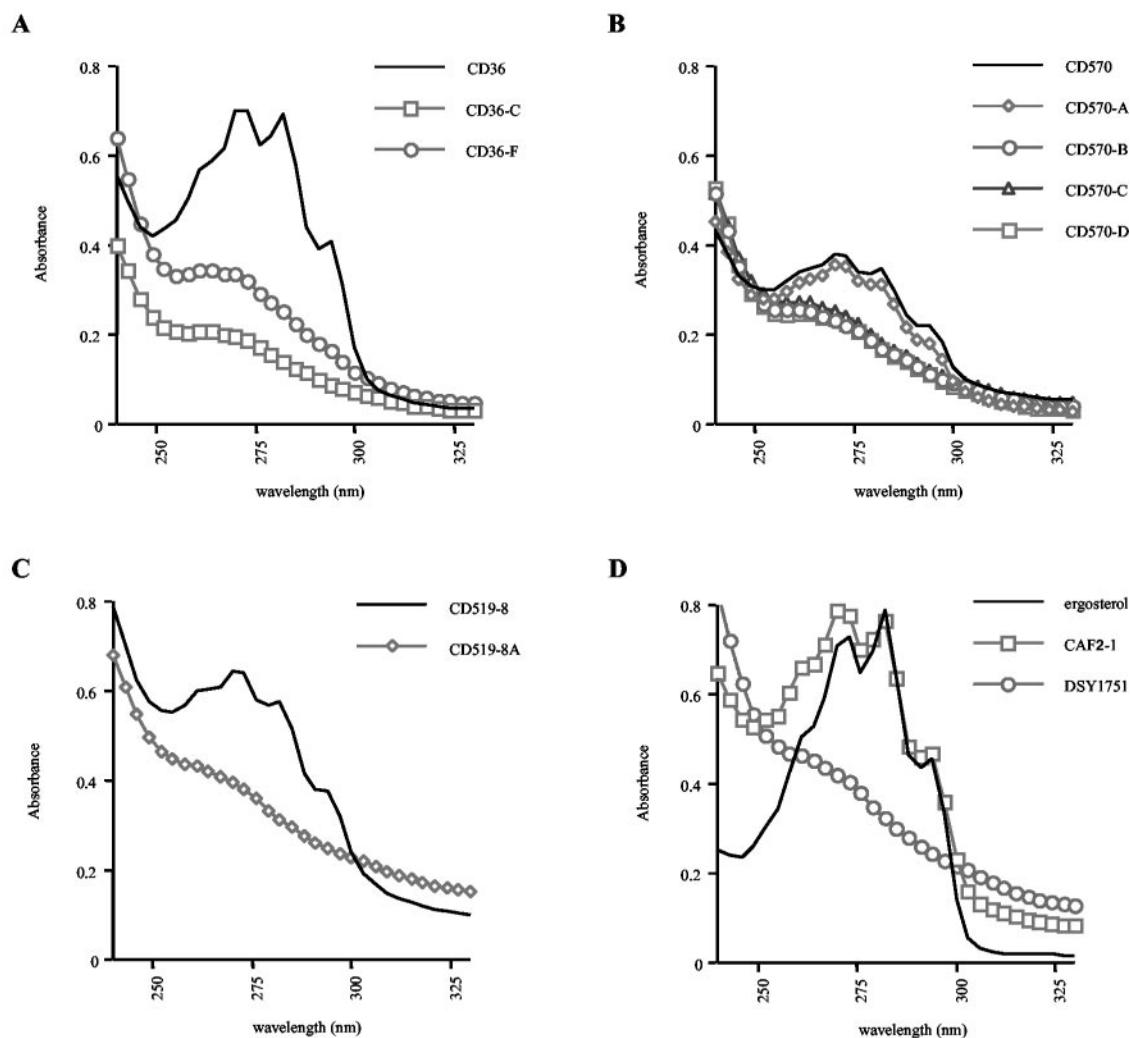


FIG. 5. UV spectrophotometric profiles of nonsaponifiable sterols from *C. dubliniensis* azole-resistant derivatives and their azole-susceptible parents. (A) CD36 and its azole-resistant derivatives CD36-C and CD36-F; (B) CD570 and its azole-resistant derivatives CD570-A, CD570-B, CD570-C, and CD570-D; (C) CD519-8 and its azole-resistant derivative CD519-8A; (D) UV spectrophotometric profiles for ergosterol and nonsaponifiable sterols from *C. albicans* wild-type (CAF2-1) and  $\Delta erg3$  (DSY1751) strains. Sterols were extracted from  $5 \times 10^8$  cells grown overnight in YEPD broth, as described in Materials and Methods.

dent clones were sequenced for each derivative. In addition, when possible (when mutations introduced or removed a restriction endonuclease site) restriction fragment length polymorphism (RFLP) analysis was carried out. Sequence analysis combined with RFLP analysis showed that all mutations identified in the derivatives, with the exception of four heterozygous mutations present in derivative CD519-8A, were homozygous. In this derivative, two distinct alleles were identified, and both of these shared the frameshift mutation.

**Expression of *CdERG3* alleles in *S. cerevisiae*.** In order to determine if the mutations identified in the azole-resistant derivatives altered the function of the sterol C5,6-desaturase enzyme, the wild-type and mutant alleles of *CdERG3* were expressed in a *S. cerevisiae*  $\Delta erg3$  strain (ATCC 4002667). First, the *CdERG3* alleles were subcloned in the yeast shuttle expression vector pYES and transformed into ATCC 4002667. In the pYES vector, the alleles were placed under the control of the galactose-inducible *GAL1* promoter. To verify that the

expression system worked, total RNA was extracted from cells growing exponentially in galactose-containing medium, and Northern blot analysis was carried out by using the 530-bp *CdERG3* amplicon described above as a probe. All transformants except the control showed high levels of expression of *CdERG3* mRNA (data not shown).

Total sterols were extracted from all transformants and were analyzed by spectrophotometry as described above. As expected, the UV absorption profile of the *S. cerevisiae*  $\Delta erg3$  strain transformed with control plasmid pYES lacked the peaks characteristic of ergosterol (Fig. 7). The absorption spectra of the transformants expressing wild-type *CdERG3* from *C. dubliniensis* parental isolate CD36, CD570, and CD519-8 alleles showed peaks at 261, 273, 282, and 294 nm, which are typical of ergosterol, indicating that these alleles were able to complement the sterol C5,6-desaturase function lacking in *S. cerevisiae*  $\Delta erg3$  since the presence of ergosterol could be detected. The sterol profiles of the transformants expressing

TABLE 3. Sterols accumulated by *C. dubliniensis* azole-resistant derivatives and their azole-susceptible parental isolates

Strain <sup>a</sup>	Accumulated sterols <sup>b</sup>
<b>CD36</b> .....	9.8% ergosta-tetraenol 64.6% ergosterol 6.8% fecosterol 8.4% obtusifoliol 10.2% unknown sterols
CD36-C.....	3.3% 7,22 isomer 73.2% ergosta 7,22 dienol 9.3% episterol 13.9% unknown sterols
CD36-F .....	4.8% 7,22 isomer 76.4% ergosta 7,22 dienol 7.0% episterol 1.3% lanosterol 10.2% unknown sterols
<b>CD519-8</b> .....	16.8% ergosta-tetraenol 55.8% ergosterol 6.7% fecosterol 6.0% episterol 1.8% obtusifoliol 12.8% unknown sterols
CD519-8A.....	8% ergostadienol 62.4% ergosta 7,22-dienol 9.2% ergostadienol 4.4% isomer of ergosterol 12.5% cholestadienol 1.6% ergosterol 1.9% unknown sterols
<b>CD570</b> .....	20% ergosta-tetraenol 60% ergosterol 4.2% fecosterol 5.8% obtusifoliol 9.9% unknown sterols
CD570-A.....	11.6% ergosta-tetraenol 55.9% ergosterol 4.1% fecosterol 6.2% obtusifoliol 22.2% unknown sterols
CD570-B.....	7.8% 7,22 isomer 61.1% ergosta-7,22-dienol 2.7% 7,22 isomer 1.4% fecosterol 7.3% episterol 1.9% ergosta-7-enol 0.8% lanosterol 16.8% unknown sterols
CD570-C.....	11.0% 7,22 isomer 57.0% ergosta-7,22-dienol 9.4% episterol 22.5% unknown sterols
CD570-D .....	7.4% 7,22 isomer 58.0% ergosta-7,22-dienol 5.4% 7,22 isomer 11.8% episterol 17.4% unknown sterols

<sup>a</sup> The parental isolates are indicated by boldface type.

<sup>b</sup> Sterols were extracted from cells grown overnight in YEPD broth at 30°C and analyzed by GC-MS, as described in Materials and Methods.

the other mutant alleles lacked the ergosterol peaks, which indicated that these mutant alleles are not able to complement the sterol C5,6-desaturase function since ergosterol was not produced by these transformants. The sterol profile of the transformant expressing the *CdERG3* allele of CD570-A (EPY64) showed peaks that were typical of those for ergosterol and that were lower than the peaks obtained with the wild-type alleles.

## DISCUSSION

*C. dubliniensis* is now well recognized as a significant human pathogen and is primarily associated with oral carriage and infection in HIV-infected individuals. Previous studies have shown that stable resistance to fluconazole can readily be induced in vitro in this species (10, 30, 31). Moreover, fluconazole-resistant *C. dubliniensis* isolates have been recovered from HIV-infected and AIDS patients treated with fluconazole for prolonged periods of time and have been shown to replace susceptible strains (36, 41). Recently, Perea and colleagues (36) showed that, as is the case in *C. albicans*, resistance to fluconazole in *C. dubliniensis* clinical isolates was associated with combinations of different molecular mechanisms such as upregulation of the multidrug resistance genes *CdCDR1* and *CdMDR1*, upregulation of *CdERG11*, and the presence of mutations in *CdErg11p*. In *C. albicans*, almost all isolates with reduced susceptibilities to azoles analyzed to date exhibit increased levels of *CDR1* expression (35). In contrast, increased levels of *CdCDR1* expression have been reported in only approximately half of the *C. dubliniensis* fluconazole-resistant isolates and derivatives analyzed to date, while increased levels of *CdMDR1* expression were reported in all *C. dubliniensis* isolates and derivatives exhibiting high-level resistance to fluconazole (30, 36). In a study that investigated the reasons for the differential regulation of *CDR1* expression in *C. albicans* and *C. dubliniensis*, Moran et al. (29) cloned and sequenced the *CDR1* gene from *C. dubliniensis*. That study showed that although *CdCDR1* is involved in reduced susceptibility to itraconazole and ketoconazole, it is not essential for fluconazole resistance in *C. dubliniensis* (29). Furthermore, 14 of 40 (35%) *C. dubliniensis* isolates studied were found to harbor nonfunctional alleles of *CdCDR1* encoding a truncated protein (29). Because of the relatively high prevalence of *C. dubliniensis* isolates harboring nonfunctional *CdCDR1* alleles in individuals receiving azole treatment, it was thought to be important to determine if the nonfunctional truncated *CdCdr1p* expressed in these isolates could affect the development of resistance to itraconazole, a triazole drug that is a substrate of *CdCdr1p* but not of *CdMdr1p* (29). Thus, the aims of the present study were to determine the incidence of itraconazole resistance in clinical isolates of *C. dubliniensis* and to investigate the mechanisms of itraconazole resistance in *C. dubliniensis* in vitro by using strains harboring either the functional or the nonfunctional alleles of *CdCDR1*.

In this study, 6 of 58 (10.4%) clinical isolates exhibited reduced susceptibility (MICs, 0.25 to 0.5 µg/ml) to itraconazole. Since the patients from whom these isolates were originally recovered had not been treated with itraconazole, it is likely that the reduced susceptibility observed was a result of fluconazole treatment. While these isolates are not considered

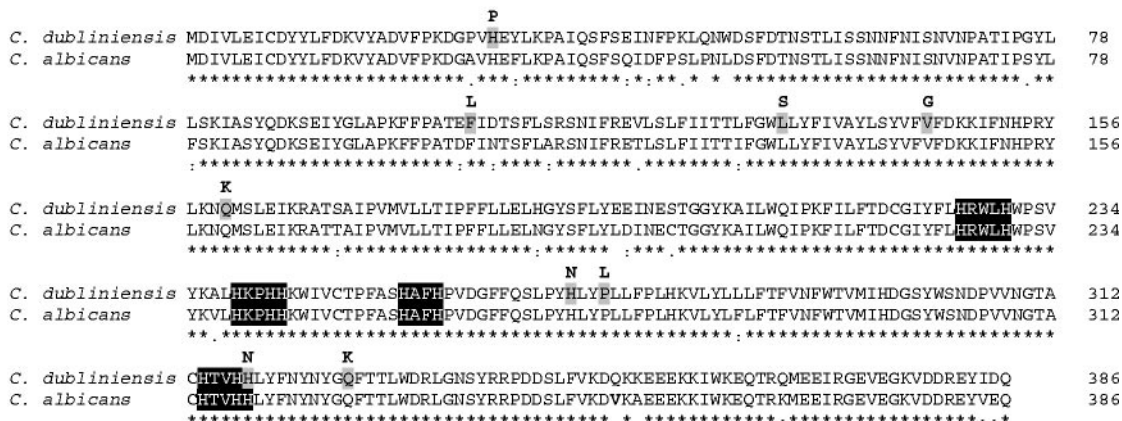


FIG. 6. Alignment of deduced amino acid sequences of sterol C5,6-desaturase in *C. dubliniensis* (EMBL accession no. AJ421248 [this study]) and *C. albicans* (GenBank accession no. AF069752 [28]) generated with the CLUSTAL W sequence alignment program (11). Asterisks indicate identical residues, colons indicate conservative substitutions, and dots indicate semiconservative substitutions. Four histidine-rich putative iron-binding domains believed to play a role in sterol C5,6-desaturase activity are highlighted in black. Amino acid mutations present in the *C. dubliniensis* azole-resistant derivatives are designated above the array. Two derivatives, CD570-B and CD519-8A, harbor frameshift mutations introducing stop codons at position 147 and 194, respectively (data not shown).

clinically resistant according to the breakpoints outlined by the National Committee for Clinical Laboratory Standards (33), they are susceptible dose dependent and the MICs for the isolates are significantly higher than those for susceptible isolates, thus indicating that resistance to itraconazole in *C. dubliniensis* may be developing clinically.

When investigating molecular mechanisms of resistance, it is preferable to use matched pairs of isolates, i.e., a drug-susceptible derivative and a drug-resistant derivative of the same strain. In the absence of matched pairs of clinical isolates,

molecular analysis of parental isolates and in vitro-generated itraconazole-resistant derivatives was used to investigate the mechanisms of itraconazole resistance in *C. dubliniensis*. In order to induce resistance to itraconazole in *C. dubliniensis*, 100 colonies each of three itraconazole-susceptible clinical isolates were sequentially exposed to increasing concentrations of the drug in agar medium up to a concentration of 64 µg/ml. The isolates selected for this study included CD36 and CD570, both of which are genotype 1 isolates and both of which harbor a nonfunctional *CdCDR1* gene, and genotype 3 isolate CD519-

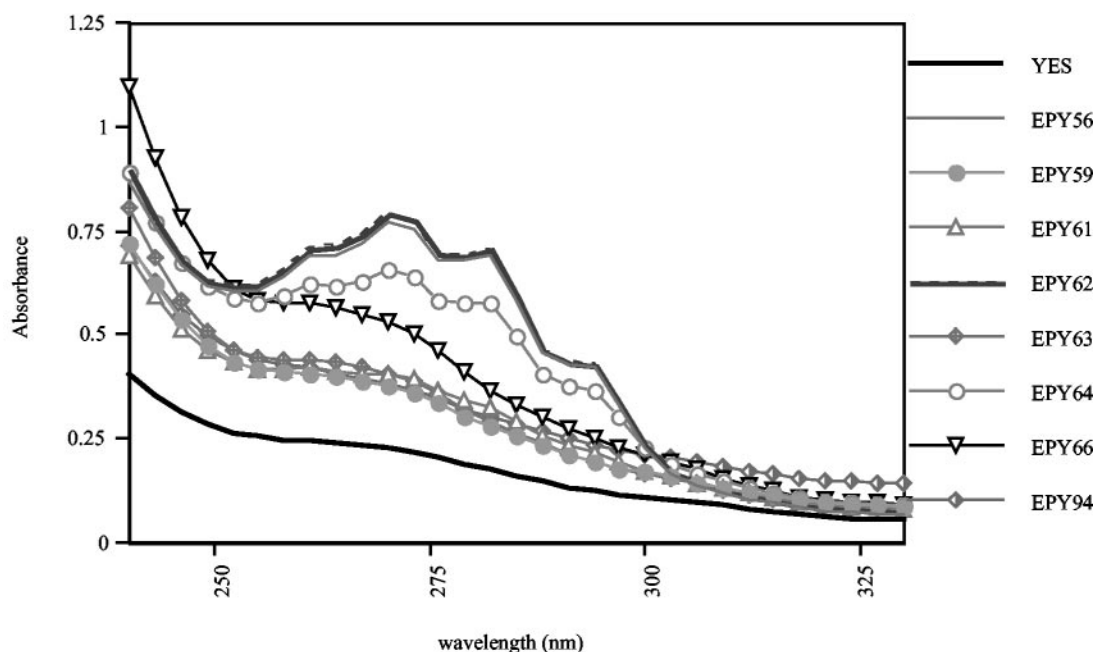


FIG. 7. UV spectrophotometric profiles of nonsaponifiable sterols extracted from the *S. cerevisiae*  $\Delta erg3$  strain expressing *CdERG3* alleles from parental isolates CD36 (EPY56) and CD570 (EPY62); derivatives CD36-C (EPY59), CD36-F (EPY61), CD570-A (EPY64), CD570-B (EPY63), CD570-C (EPY66), and CD519-A (EPY94); and a control transformed with the expression vector pYES (YES). Total sterols were extracted from  $5 \times 10^8$  cells grown overnight in galactose-containing defined broth medium as described in Materials and Methods.

8, which was chosen because it harbored a functional *CdCDR1* gene. Exposure to itraconazole resulted in the recovery of seven stable itraconazole-resistant derivatives which exhibited significantly reduced susceptibilities to itraconazole (MIC range, 32 to 64  $\mu\text{g/ml}$ ). These derivatives were found to exhibit cross-resistance to fluconazole (MIC range 128 to 264  $\mu\text{g/ml}$ ) and ketoconazole (MIC range, 1 to 4  $\mu\text{g/ml}$ ). Six of the derivatives exhibited reduced susceptibilities to amphotericin B (MIC range, 0.06 to 0.25  $\mu\text{g/ml}$ ). In contrast, no cross-resistance to inhibitors or other antifungal drugs was observed (Table 2). Indeed, the derivatives were slightly more susceptible to these agents than their respective parental isolates.

In order to identify the molecular mechanisms responsible for the derivatives' azole-resistant phenotype, the most common mechanisms of azole resistance in *Candida* species were investigated. All derivatives exhibited an increased level of *CdERG11* expression of at least fourfold. While overexpression of *ERG11* has been described in several different azole-resistant clinical isolates of *Candida* species (36, 46, 53), this has always been accompanied by other modifications associated with azole resistance and the effects of overexpression of *ERG11* have not been extensively evaluated. Thus, it was thought to be unlikely that upregulation of *CdERG11* alone was responsible for the azole-resistant phenotypes of the derivatives.

Changes in the amino acid sequence of CdErg11p affecting the affinity of this enzyme for azole antifungal agents is a well-described mechanism of azole resistance. However, comparative analysis of the nucleotide sequences of *CdERG11* genes from the derivatives and their respective parental isolates did not reveal specific mutations in the derivatives that could be associated with azole resistance.

Because the derivatives exhibited pan-azole resistance but did not exhibit cross-resistance to inhibitors which are normally substrates of multidrug resistance proteins, the involvement of these proteins in resistance seemed unlikely. Nevertheless Northern blot analysis was carried out to investigate levels of expression of the *CdCDR* and *CdMDR1* genes. As expected, overexpression of *CdMDR1* did not appear to be involved in the itraconazole-resistant phenotype, as itraconazole is not a substrate of CdMdr1p. There was, however, a noticeable increase (from 2.5- to 12-fold) in the level of expression of *CdCDR1* in derivatives CD36-C, CD36-F, CD570-B, CD570-C, CD570-D, and CD519-8A but not in derivative CD570-A. Overexpression of *CdCDR2* was not detected in any derivative (Fig. 2). In order to determine if increased levels of expression of multidrug resistance genes resulted in increased drug efflux in the azole-resistant derivatives, the glucose-mediated efflux of R6G was measured. The results obtained suggested that R6G efflux mediated by drug extrusion pumps was less effective in derivatives CD36-C, CD36-F, CD570-B, CD570-C, CD570-D, and CD519-8A than in their parental isolates. This was not the case for derivative CD570-A, for which R6G efflux was similar to that for its parental strain, CD570. These data suggest that the membranes of derivatives CD36-C, CD36-F, CD570-B, CD570-C, CD570-D, and CD519-8A have altered properties, possibly as a result of alterations in the ergosterol biosynthetic pathway. It has previously been demonstrated that altered membrane content can affect the function of efflux pumps (15, 32, 47). Resistance to

azole antifungal agents due to alterations of the ergosterol biosynthetic pathway has been documented in *S. cerevisiae* (16) and in clinical isolates of *C. albicans* (18, 19). In these studies, a defect in sterol C5,6-desaturase was identified as the mechanism causing resistance to azoles. In C5,6-desaturase-deficient cells, growth in the presence of azoles is supported by the accumulation of 14 $\alpha$ -methyl-fecosterol instead of the toxic metabolite 14 $\alpha$ -methyl-3,6-diol that normally accumulates during azole treatment, with the accumulation ultimately causing growth arrest. To determine if the derivatives were deficient in sterol C5,6-desaturase, the sterol contents of their membranes were extracted and analyzed. Derivatives CD36-C, CD36-F, CD570-B, CD570-C, CD570-D, and CD519-8A did not produce ergosterol, which was consistent with a lack of sterol C5,6-desaturase activity in these derivatives. This was not the case, however, for derivative CD570-A, which contained ergosterol. In order to identify mutations in the gene encoding sterol C5,6-desaturase (termed *ERG3*), it was necessary to clone the *C. dubliniensis* *ERG3* gene, which was found to be highly homologous (91.1%) to its *C. albicans* counterpart. Sequence analysis of the *CdERG3* genes of the derivatives revealed that all derivatives (including CD570-A) harbored at least one homozygous mutation which altered the amino acid sequence of CdErg3p.

Heterologous expression studies revealed that these mutations resulted in a loss of Erg3p function for six of the seven azole-resistant derivatives. Mutated alleles of *CdERG3* (with the exception of derivative CD570-A) were unable to complement the defective sterol C5,6-desaturase function in an *S. cerevisiae*  $\Delta\text{erg3}$  mutant, whereas wild-type parental alleles could restore this function.

While loss-of-function mutations in the *CdERG3* gene were observed in six derivatives exhibiting azole resistance, this was not the case for derivative CD570-A. This derivative exhibited levels of azole resistance which were similar to those for the other six derivatives and, like them, did not show cross-resistance to inhibitors. Unlike the other six derivatives, CD570-A did not exhibit reduced susceptibility to amphotericin B. Like the other six derivatives, derivative CD570-A exhibited elevated levels of expression of *CdERG11*, and there were no changes in the predicted amino acid sequence of CdErg11p which could explain its azole resistance phenotype. In contrast to the other six derivatives, the level of *CdCDR1* expression was not elevated in CD570-A and R6G efflux did not appear to be altered in this derivative. Although derivative CD570-A accumulated ergosterol in its membranes like its parental isolate, there was a noticeable difference in the overall membrane sterol contents between CD570-A and CD570. The presence of a mutation in CdErg3p seemed to have little effect on the activity of the sterol C5,6-desaturase of CD570-A, as the heterologous expression of the CD570-A *CdERG3* gene was able to complement the defective sterol C5,6-desaturase activity of an *S. cerevisiae*  $\Delta\text{erg3}$  strain. It is unlikely that overexpression of *CdERG11* is the only mechanism responsible for azole resistance in derivative CD570-A. While the reasons for this resistance in derivative CD570-A are as yet undetermined, the observed changes in membrane sterol contents could suggest that unidentified alterations of the ergosterol biosynthetic pathway could contribute to the azole-resistant phenotype in this derivative of CD570.

Although the derivatives showed increased levels of *CdCDR1* and *CdERG11* expression, the data presented in this study are consistent with a loss of function of *CdERG3* as the primary mechanism for itraconazole resistance in six of the seven derivatives. The observed overexpression of *CdERG11* in all derivatives, although not the primary mechanism, may be a factor contributing to their azole resistance. However, loss-of-function mutations of *ERG3* mediate azole resistance by exerting a suppressor effect on yeast cells lacking 14 $\alpha$ -lanosterol demethylase activity (52). Therefore, the observed overexpression of the demethylase is likely to be both unnecessary and irrelevant for azole resistance in the derivatives with loss-of-function mutations in *CdERG3*. Moreover, the observed upregulation of *CdERG11* is very likely a consequence of the absence of ergosterol in the derivatives lacking sterol C5,6-desaturase activity. Indeed, such a negative-feedback effect on the regulation of *ERG11* expression has previously been observed in a *C. glabrata*  $\Delta$ erg3 strain (9).

Since two of the parental isolates used in the present study were chosen because they harbored nonfunctional *CdCDR1* genes, overexpression of *CdCDR1* in their azole-resistant derivatives should have no effect on azole resistance. It is somewhat surprising that derivatives harboring nonfunctional *CdCDR1* genes showed increased *CdCDR1* mRNA levels. Indeed, data from this study suggest a correlation between a lack of sterol C5,6-desaturase activity and overexpression of *CdCDR1* by azole-resistant derivatives. It is conceivable that changes in membrane lipid composition affect factors involved in regulating *CdCDR1* expression, as it has been proposed that Cdr1p may be involved in phospholipid translocation (2).

The results from this study show that mutations in sterol C5,6-desaturase are involved in high-level stable azole resistance following exposure to itraconazole in vitro. The *C. albicans* Darlington strain is an example of a highly resistant clinical strain (itraconazole MIC, >4  $\mu$ g/ml; fluconazole MIC, 32  $\mu$ g/ml) harboring, as well as other resistance mechanisms, mutations in sterol C5,6-desaturase (28). This suggests that the levels of azole resistance and the resistance mechanisms observed in the azole-resistant derivatives of *C. dubliniensis* are of clinical relevance. While it has previously been established that multiple factors contribute to the development of resistance in *Candida* species, the findings of this study highlight the fact that, if not thoroughly investigated, azole resistance can wrongly be attributed to the apparent overexpression of multidrug resistance genes and of the target enzyme *ERG11*. Indeed, more studies are needed to evaluate the contribution of sterol C5,6-desaturase loss-of-function mutations in clinical azole resistance.

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