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The *Candida dubliniensis CdCDR1* Gene Is Not Essential for Fluconazole Resistance

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The present study investigated the role of the *Candida dubliniensis CdCDR1* and *CdCDR2* genes in the development of fluconazole resistance. The *C. dubliniensis CdCDR1* gene was 92% identical at the nucleotide sequence level to the corresponding *C. albicans* gene. However, 58% (14 of 24) of *C. dubliniensis* genotype 1 isolates tested harbored a nonsense mutation in the *CdCDR1* open reading frame that converted codon 756 (TAT) to a TAG translational stop codon. Analysis of five of these *C. dubliniensis* isolates by Western immunoblotting showed that they expressed a truncated 85-kDa CdCdr1p compared to the full-length 170-kDa CdCdr1p. Expression of *CdCDR1* alleles from six *C. dubliniensis* isolates in a *pdr5 Saccharomyces cerevisiae* strain revealed that *CdCDR1* alleles from three isolates that encoded truncated proteins were unable to confer resistance to drugs and antifungals. However, reassignment of the TAG sequence at codon 756 to TAT (encoding tyrosine) in an allele from strain CD36 conferred the ability to mediate resistance to multiple drugs. Fluconazole-resistant isolates of *C. dubliniensis* harboring functional alleles of *CdCDR1* were found to exhibit two- to ninefold-higher levels of *CdCDR1* mRNA than did matched fluconazole-susceptible isolates. By comparison, levels of *CdMDR1* expression ranged from approximately 50- to 100-fold greater in resistant isolates. Fluconazole resistance was also identified in isolates harboring nonfunctional *CdCDR1* alleles, but resistance in these isolates was only associated with increased *CdMDR1* expression. Targeted disruption of two functional alleles of *CdCDR1* in a fluconazole-resistant derivative of *C. dubliniensis* that overexpressed both *CdCDR1* and *CdMDR1* revealed that although *CdCDR1* was important for mediating reduced susceptibility to itraconazole and ketoconazole, there was no affect on fluconazole susceptibility in the double mutant. Evidence presented in this study reveals that *CdCDR1* is not essential for the development of fluconazole resistance in *C. dubliniensis*.

Resistance to azole antifungal drugs in *Candida* species is now recognized as a major clinical problem (17, 32). Several studies have shown that a significant proportion of oral *Candida albicans* isolates recovered from human immunodeficiency virus (HIV)-infected patients are resistant to the azole antifungal drug fluconazole and that some non-*C. albicans Candida* species, such as *Candida krusei,* are inherently resistant to this agent (1, 9, 11, 22). Fluconazole resistance has also been described in *Candida dubliniensis*, a species that was first described in 1995 (14, 30). *C. dubliniensis* is a significant cause of oral disease in the HIV-infected patient population, who routinely receive fluconazole therapy for the suppression of oral candidiasis (4, 29). Recently, several studies have reported the recovery of *C. dubliniensis* from the bloodstream, although the true incidence of systemic infection caused by this organism has yet to be determined (2, 3, 12). We and others have previously reported the recovery of *C. dubliniensis* isolates with reduced susceptibility to fluconazole from the oral cavities of HIV-infected patients (10, 13, 14, 20). In addition, we have shown that fluconazole-susceptible *C. dubliniensis* clinical isolates can readily develop fluconazole resistance when exposed to this agent in vitro (14). Molecular analysis of fluconazole-resistant *C. dubliniensis* isolates and in vitro-generated fluconazole-resistant derivatives has shown that in each case, the fluconazole resistance phenotype is associated with increased expression of the *CdMDR1* gene encoding the multidrug transporter CdMdr1p (13). Wirsching et al. demonstrated that targeted deletion of both copies of *CdMDR1* in a *C. dubliniensis* clinical isolate with reduced susceptibility to fluconazole was sufficient to render the null mutant susceptible to fluconazole (35).

A second multidrug transporter-encoding gene, termed *CdCDR1*, has also been identified in *C. dubliniensis*, and it is homologous to the *C. albicans* ABC transporter-encoding gene *CDR1* (13). Almost all isolates of *C. albicans* (83%) with reduced susceptibility to azoles analyzed to date exhibit increased expression of *CDR1* (16). However, the role of the homologous *CdCDR1* gene in *C. dubliniensis* in determining susceptibility to fluconazole is less clear. Although increased expression of *CdMDR1* has been observed in all fluconazole-resistant *C. dubliniensis* isolates and derivatives analyzed to date, increased *CdCDR1* gene expression has only been reported in approximately 50% of fluconazole-resistant *C. dubliniensis* isolates and derivatives (13). For these reasons, the objectives of the present study were to functionally characterize the *C. dubliniensis CdCDR1* gene and to examine its role in determining azole susceptibility in *C. dubliniensis*.

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MATERIALS AND METHODS

Candida strains and culture conditions. Forty clinical isolates of *C. dubliniensis* from diverse geographic locations were included in this study (Table 1). Isolates of *C. dubliniensis* were routinely cultured on potato dextrose agar (Ox-

TABLE 1. *Candida* strains and derivatives used in this study

Strain or isolate	Source and/or notes[a]	Country of origin	TAG[c]	Reference(s)
C. albicans CA132A	Oral reference isolate	Ireland	NA[b]	7
C. dubliniensis				
Genotype I				
CD36	Type strain, CBS 7987	Ireland	+	30
CD36R1	Derivative of CD36, FLU^{DD}	NA	+	8
CD36R2	Derivative of CD36, Flu^r	NA	+	8
CM1	Oral isolate	Australia	−	14, 30
CM2	Oral isolate, FLU^{DD}	Australia	−	14, 30
CD57	Vaginal isolate	Ireland	−	14
CD57R	Derivative of CD57, FLU^{DD}	NA	−	14
57RM1	*CdCDR1*/*cdcdr1*Δ::MPA^R-*FLIP*	NA	−	This study
57RM2	*CdCDR1*/*cdcdr1*Δ::*FRT*	NA	−	This study
57RM3	*cdcdr1*Δ::MPA^R-*FLIP*/*cdcdr1*Δ::*FRT*	NA	NA	This study
57RM4	*cdcdr1*Δ::*FRT*/*cdcdr1*Δ::*FRT*	NA	NA	This study
CD51-II	Oral isolate	Ireland	+	14
CD51-IIR	Derivative of CD51-II, Flu^r	NA	+	14
CD47-I	Oral isolate	Ireland	+	14
CD47-IIa	Oral isolate	Ireland	+	14
CD47-IIb	Oral isolate, FLU^{DD}	Ireland	+	14
CD33	Oral isolate	Ireland	−	30
CD38	Oral isolate	Ireland	+	30
CD71	Oral isolate	Argentina	−	8
CD72	Oral isolate, Flu^r	Ireland	−	14
NCPF3108	Postmortem lung isolate	United Kingdom	+	8, 30
CBS8500	Blood culture isolate	The Netherlands	−	12
CM5	Oral isolate	Australia	−	30
CO4	Oral isolate	Switzerland	+	8
P30	Oral isolate	Switzerland	+	8
CD96.34	Oral isolate	Germany	±	8
CD159	Oral isolate	Greece	−	8
CAN1	Oral isolate	Canada	+	8
Wü284	Oral isolate	Germany	+	27
B341	Oral isolate	United States	+	This study
B484	Oral isolate	United States	−	This study
B651	Oral isolate	United States	±	This study
Genotype II				
CBS2747	Sputum isolate	The Netherlands	−	8
CAN3	Oral isolate	Canada	−	8
CAN4	Oral isolate	Canada	−	8
CAN6	Oral isolate	Canada	−	8
CD41	Oral isolate	Ireland	−	14
m262b	Oral isolate	United Kingdom	−	8
CD513	Oral isolate	Ireland	−	8
CD506	Oral isolate	Ireland	−	8
CD539	Oral isolate	United Kingdom	−	8
CD541	Blood culture isolate	United Kingdom	−	8
Genotype III				
p7276	Respiratory tract isolate	Israel	−	18
p6265	Sputum isolate	Israel	−	18
p6785	Urine isolate	Israel	−	18
CD514	Oral isolate	Ireland	−	8
CD519	Oral isolate	Ireland	−	8
Genotype IV				
p7718	Wound isolate	Israel	−	18

[a] Isolates are fluconazole susceptible (MIC, ≤8 μg/ml) unless indicated. Flu^r, fluconazole resistant (MIC, ≥64 μg/ml); FLU^{DD}, dose-dependent fluconazole resistance (MIC, 16 to 32 μg/ml).

[b] NA, not applicable.

[c] Refers to presence (+) or absence (−) of a TAG at codon 756 of the *CdCDR1* ORF; ± refers to heterozygosity at this locus.

oid) medium, pH 5.6, at 37°C (7). For liquid culture, cells were grown in yeast extract-peptone-dextrose (YEPD) broth, also at 37°C.

Transformants of *C. dubliniensis* and *Saccharomyces cerevisiae* were selected and maintained on minimal agar medium (6.7 g of yeast nitrogen base without amino acids [Difco], 20 g of glucose, 15 g of Bacto agar [Difco], and 50 mg [each] of uracil, lysine, adenine, tryptophan, and histidine [Sigma-Aldrich] per liter). For induction of the *SAP2* promoter and excision of the mycophenolic acid (MPA) resistance flipper cassette (MPA^R-flipper), cells were grown in YCB-BSA medium (23.4 g of yeast carbon base [Difco], 4 g of bovine serum albumin [Sigma-Aldrich] per liter [pH 4.0]).

TABLE 2. Oligonucleotide primers used in PCRs

Gene and primer	Nucleotide sequence (5′–3′)[a]	Nucleotide coordinates[b]
CdCDR1		
1F	AATCTAGAATTATGTCAGATTCTAAGATGTC	−3 to +20
1R	ATTCTAGATTATTTCTTATTTTTTTTCTCTCTG	+4482 to +4506
2F	ATCCTGTTGGTTATGTGTTCG	+2071 to +2091
2R	GGGAAATCAACACTTCCAGTG	+2557 to +2537
3F	AGGAATTCCACTTGTGTGGGCATCAAACC	+121 to +101
3R	AGGAATTCCCAATGTTGGGATAATGCCAC	+969 to +989
4F	AGGAATTCAAACCTGGGCCACTTGGAAC	+2165 to +2146
4R	AGGAATTCCATTGTACAGTGAAAGATGGAG	+4373 to +4394
5F	ACCGCTCGAGCAGGTCTCATGATAGCATCC	+538 to +519
5R	ATCCCCGCGGAACTTGTTCTAGTTATTTGG	+4248 to +4267
6F	ATCCCCGCGGCAATACTTGTTGGTCAGTTGG	+3532 to +3552
6R	ACCGCTCGAGATATGGAGATGCTGGTCTGG	+1467 to +1448
7R[c]	AATCTAGAGTTGATAATCAGAATCATTAGC	+421 to +400
CdCDR2		
8F	AATCTAGAAATATGAGTACTGCAAACACGTC	−3 to +20
8R	ATTCTAGATTATTTTTTCATCTTCTTTTCTCTATT	+4503 to +4467
9F	GCGAATTCAAACCTTGATATTCTTGAACTG	+97 to +76
9R	GCGAATTCGTTGGGATAATGCCACTAG	+989 to +971
10F	GCGAATTCTAATGCTTCTCTAACAGTAAGTGG	+2862 to +2840
10R	GCGAATTCCTATGGGACAATTGGCAATC	+3986 to +4005
7R[c]	AATCTAGAGTTGATAATCAGAATCATTAGC	+418 to +394
TEF3[d]		
TEF3F	CGGAATTCCGATTGGTCCAAATGGTGCTGG	+2113 to +2132
TEF3R	CGGAATTCCGATCTTGTTACCCATAGCATCG	+3012 to +3032

[a] Restriction endonuclease recognition sequences are underlined. *Xba*I, 5′-TCTAGA; *Eco*RI, 5′-GAATTC; *Xho*I, 5′-CTCGAG; *Sac*II, 5′-CCGCGG.
[b] Nucleotide coordinates for *CdCDR1* (accession no. AJ439073) or *CdCDR2* (accession no. AJ439075) are as indicated, with the first base of the ATG start codon designated + 1.
[c] The primer 7R is homologous to both *CdCDR1* and *CdCDR2*.
[d] Nucleotide coordinates of the *TEF3* primers refer to the *C. albicans TEF3* gene (accession no. Z12822).

Antifungal drug susceptibility testing. Susceptibility testing of *C. dubliniensis* clinical isolates and their derivatives to antifungal drugs and metabolic inhibitors was performed by using a broth microdilution assay based on the approved NCCLS procedure (14). Susceptibility tests were carried out in RPMI 1640 medium (10.4 g of RPMI 1640 [Sigma-Aldrich], 20 g of glucose, 34.5 g of morpholinepropanesulfonic acid [pH 7.0] per liter) as described by Moran et al. (14). Metabolic inhibitors were purchased from Sigma-Aldrich, fluconazole was a gift from Pfizer Central Research (Sandwich, Kent, United Kingdom), and itraconazole and ketoconazole were gifts from Janssen Pharmaceutical (Cork, Republic of Ireland).

The following antifungal drugs and metabolic inhibitors were prepared as stock solutions in water at the concentrations indicated: fluconazole, 5 mg/ml; fluphenazine, 20 mg/ml; and rhodamine 6G, 5 mg/ml. Stock solutions of other drugs were prepared in dimethyl sulfoxide at the concentrations indicated: ketoconazole, 1 mg/ml; itraconazole, 1 mg/ml; cycloheximide, 20 mg/ml; cerulenin, 1 mg/ml; and brefeldin A, 5 mg/ml. Crystal violet (2 mg/ml) was dissolved in methanol. Stock solutions of drugs were diluted in RPMI 1640 medium to the following concentrations, from which serial twofold dilutions were prepared in 96-well microtiter dishes (Corning): fluconazole, 64 μg/ml; itraconazole, 4 μg/ml; ketoconazole, 2 μg/ml; cerulenin, 64 μg/ml; rhodamine 6G, 16 μg/ml; cycloheximide, 512 μg/ml; brefeldin A, 512 μg/ml; and fluphenazine, 128 μg/ml.

PCR amplification of *C. dubliniensis CdCDR1* alleles. Amplification of *CdCDR1* and *CdCDR2* genes was carried out by using the Expand high-fidelity PCR system (Roche Molecular Biochemicals). To amplify the entire *C. dubliniensis CdCDR1* open reading frame (ORF), the oligonucleotide primer pair 1F-1R (Table 2; Fig. 1) was designed based on the nucleotide sequence of the *C. albicans CDR1* gene. Similarly, for amplification of the *CdCDR2* ORF, the primer pair 8F-8R was designed based on the nucleotide sequence of the *C. albicans CDR2* gene. These primers contained cleavage sites for the restriction endonuclease *Xba*I (Table 2). Template DNA was isolated from *C. dubliniensis* isolates as described by Sullivan et al. (28). Sequences flanking the *CdCDR1* and *CdCDR2* genes were amplified from the *C. dubliniensis* type strain CD36 by inverse PCR. Briefly, PCR amplification was carried out under the same conditions with self-ligated *Eco*RI-digested total genomic DNA as template. The 5′ flanking region of the *CdCDR1* gene was amplified with the oligonucleotide primer pair 3F-3R, which contained the *Eco*RI restriction endonuclease recognition sequence (Table 2; Fig. 1). The 3′ region of the *CdCDR1* gene was amplified with the oligonucleotide primer pair 4F-4R (Table 2; Fig. 1). Similarly, the 5′ and 3′ flanking sequences of the *CdCDR2* gene were amplified with the oligonucleotide primer pairs 9F-9R and 10F-10R, respectively. PCR products were purified from the reaction mixture by using the Wizard PCR system (Promega). PCR primers were designed with restriction endonuclease recognition sequences at their 5′ ends (Table 1) that allowed PCR products to be digested and ligated directly to the plasmid vector pBluescript II KS(−), transformed into *Escherichia coli* DH5α as described previously, and subjected to DNA sequence analysis (21).

Heterologous expression of *CdCDR* genes in *S. cerevisiae*. The oligonucleotide primer pairs 1F-1R and 8F-8R (Table 2; Fig. 1) were used to amplify the entire *CdCDR1* and *CdCDR2* ORFs, respectively. The *CdCDR1* and *CdCDR2* ORFs were amplified from genomic DNA purified from *C. dubliniensis* isolates CD36, CD57, CD51-II, CD47-IIb, CM1, and CM2 and *C. albicans* strain CA132A. These products were cloned by using standard techniques into the *Xba*I site of the expression plasmid pYES (15) and transformed into the Δ*pdr5 S. cerevisiae* strain YKKB-13 as described by Sanglard et al. (24). Transformants of YKKB-13 were selected on minimal medium without uracil. In order to induce expression from the *GAL1* promoter of pYES, transformants were subsequently maintained on minimal medium without uracil containing 2% (wt/vol) galactose as the sole carbon source. *S. cerevisiae* transformants were tested for susceptibility to antifungal drugs and metabolic inhibitors on minimal medium containing galactose as described by Sanglard et al. (24). Briefly, a suspension (2×10^7 CFU/ml) of each transformant to be tested was prepared in sterile saline. This solution was then serially diluted 10-fold, and 5 μl of each dilution was spotted onto plates containing fixed concentrations of each metabolic inhibitor (see Fig. 3). Susceptibility to each drug was determined based on the highest dilution of each culture which could grow in the presence of the inhibitor as described by Sanglard et al. (24).

Western immunoblotting. Crude protein extracts were prepared from *C. dubliniensis* isolates, resolved by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), and subjected to Western immunoblot analysis with anti-Cdr1p polyclonal sera (a gift from D. Sanglard) as described by Moran et al.

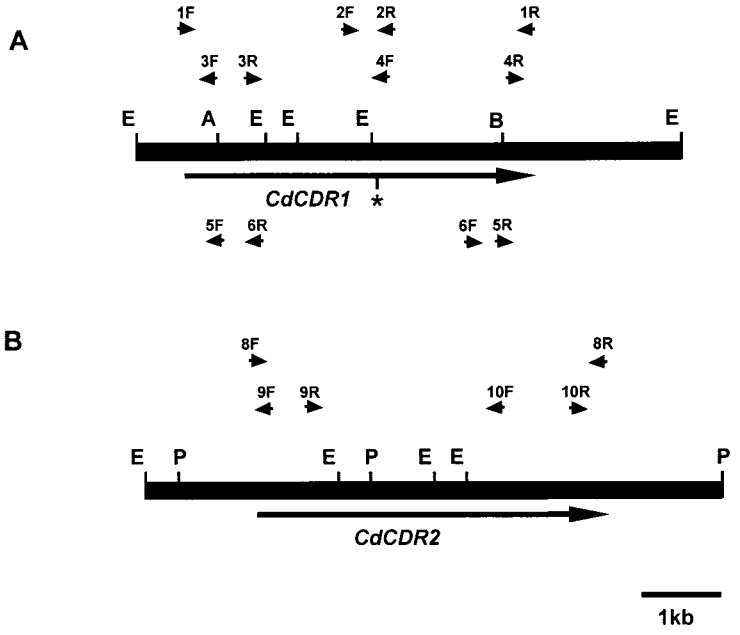

FIG. 1. Restriction map of *CdCDR1* (A) and *CdCDR2* (B) encoding DNA from *C. dubliniensis* CD36. The black rectangular boxes represent *C. dubliniensis* genomic DNA which was amplified by PCR from genomic DNA (see Materials and Methods). The large arrows show the positions and directions of transcription of the *CdCDR1* and *CdCDR2* ORFs. The asterisk in the *CdCDR1* ORF shows the position of the polymorphic codon 756. The approximate positions of the *CdCDR1*- and *CdCDR2*-specific PCR primers shown in Table 1 are indicated by small arrows. Primer pairs 5F-5R and 6F-6R shown at the bottom of panel A were used in the inverse PCR experiments performed to disrupt the *CdCDR1* gene. Restriction endonuclease cleavage sites: A, *Acc*I; B, *Bam*HI; E, *Eco*RI; P, *Pvu*II.

(13). Antibody-protein complexes were detected with the SuperSignal West Pico chemiluminescent substrate (Pierce, Rockford, Ill.). Molecular sizes of protein bands were estimated by using the Bio-Rad broad-range SDS-PAGE standards (Bio-Rad) and the GelWorks one-dimensional gel analysis software package (UVP, Irvine, Calif.).

Site-directed mutagenesis. Site-directed mutagenesis was carried out by using the GeneEditor system (Promega). Alleles of *CdCDR1* for mutagenization were subcloned into the plasmid vector pGEM-11Z(f+). Point mutations were introduced by hybridization with the 5′-phosphorylated mutagenic oligonucleotide 5′-GCTTACCAATATTATAATTC, which contained a single G to T substitution (underlined), according to the manufacturer's instructions. Mutant alleles were sequenced on both strands in order to confirm the introduction of the desired mutation.

Isolation of total RNA from *C. dubliniensis* and Northern blot analysis. Total RNA was isolated from *C. dubliniensis* isolates grown to the mid-exponential phase (optical density at 600 nm, 0.6) in YEPD broth at 37°C as described by Moran et al. (13). RNA was fractionated on 1.2% (wt/vol) agarose gels containing 6% (vol/vol) formaldehyde and transferred to nylon membranes (Osmonics, Westborough, Mass.) by capillary transfer. RNA was hybridized at 42°C with DNA probes homologous to *CdCDR1*, *CdMDR1*, and *CdTEF3* labeled with [α-^{32}P]dATP (6,000 Ci/mmol, 220 TBq/mmol; NEN Life Sciences, Boston, Mass.) by random primer labeling as described by Moran et al. (13). A *CdCDR1*-specific probe was constructed by PCR amplification of the 5′ end of the *CdCDR1* gene (nucleotides +1 to +421) with the primer pair 1F-7R (Table 2). Similarly, a 418-bp *CdCDR2*-specific probe (nucleotides +1 to +418) was amplified from *C. dubliniensis* genomic DNA by using the primer pair 8F-7R (Table 2). A *CdTEF3*-specific probe was created by amplification of an internal fragment of the *CdTEF3* gene with the primer pair TEF3F-TEF3R (Table 2). Signals from Northern blot autoradiograms were quantified by scanning densitometry and normalized for loading against the *CdTEF3* expression signal with the GelWorks one-dimensional software package (UVP).

Targeted disruption of the *CdCDR1* gene. Targeted disruption of the *CdCDR1* gene in *C. dubliniensis* CD57R was carried out by using the *MPA*^{R}-flipper technique (34, 35). Two different deletion constructs were made to inactivate both alleles of the *CdCDR1* gene. To create the first construct, the entire *CdCDR1* ORF was cloned in the *Xba*I site of pUC19. A deletion was created in this clone by inverse PCR with the primer pair 5F-5R (Table 2; Fig. 1). This allowed amplification of the 5′ and 3′ ends of the *CdCDR1* ORF and the entire plasmid vector (21). These primers contained *Sac*II and *Xho*I restriction endonuclease recognition sequences that enabled the insertion of the *Sac*II/*Xho*I fragment of pSFI1 (containing the site-specific recombinase FLP and the MPA resistance gene) (34, 35), thereby replacing the central region of the *CdCDR1* ORF to create plasmid pCDRΔ1. To create the second construct, the internal *Acc*I/*Bam*HI fragment of *CdCDR1* was cloned into pUC19 and subjected to inverse PCR with the primer pair 6F-6R (Table 2; Fig. 1). This product was also ligated with the *Sac*II/*Xho*I fragment from pSFI1 to create the plasmid pCDRΔ2. *C. dubliniensis* was sequentially transformed by using approximately 1 μg of the linear *Sac*I/*Sph*I fragments from plasmids pCDRΔ1 and pCDRΔ2 by electroporation as described by Staib et al. (27). MPA-resistant transformants were selected on minimal medium agar plates containing 10, 15, or 20 μg of MPA per ml.

Isolation of genomic DNA and Southern blotting. Genomic DNA from *C. dubliniensis* isolates and derivatives for use in PCR and Southern blotting experiments was isolated by using the method of Sullivan et al. (28). For Southern blotting, 10 μg of genomic DNA was digested with the appropriate restriction endonuclease and separated on 0.8% (wt/vol) agarose gels. DNA was transferred to nylon membranes (Osmonics) by capillary transfer overnight. Hybridization was carried out under high-stringency conditions by standard techniques with DNA probes labeled with α-^{32}P by random primer labeling (28).

RESULTS

Isolation and sequence analysis of *CdCDR1*. In order to isolate the *C. dubliniensis* homologue of the *C. albicans CDR1* gene, a 6,848-bp region of *C. dubliniensis* chromosomal DNA was PCR amplified by using a high-fidelity polymerase mixture from genomic DNA isolated from the *C. dubliniensis* type strain CD36 (CBS 7987). Initially, the primer pair 1F-1R (Table 2; Fig. 1), which was designed to amplify the entire *C. albicans CDR1* ORF, was used to amplify a 4,506-bp region of *C. dubliniensis* genomic DNA corresponding to the putative *C.*

dubliniensis CDR1 homologue. The nucleotide sequences 5′ and 3′ to this region were then amplified from *C. dubliniensis* CD36 genomic DNA by an inverse PCR method with the primer pairs 3F-3R and 4F-4R (Table 2; Fig. 1), respectively. Using this strategy, a 614-bp region upstream of the putative *CdCDR1* ORF and a 1,728-bp downstream region were amplified from *C. dubliniensis* CD36 chromosomal DNA. The entire amplified chromosomal DNA region from strain CD36 was then sequenced on both strands. The putative *CdCDR1* ORF amplified with the primer pair CDR1F-CDR1R was 4,506 bp in length and was 92% identical at the nucleotide sequence level to the *C. albicans CDR1* ORF. The 614-bp 5′-flanking region of this sequence shared 46.6% identity at the nucleotide sequence level with the *C. albicans CDR1* promoter and contains a putative TATA box at nucleotide position −126 (the first base of the ATG start codon was designated +1). In addition, the *C. dubliniensis* promoter sequence contains a motif at the nucleotide coordinates −371 to −350 (5′-CGGT TATCGGATATTTTTTTT) matching the drug response element (DRE) in *C. albicans* (5). The 3′-flanking region of the putative *CdCDR1* ORF also contained sequences homologous to the *C. albicans SAP3* gene encoding a member of the secreted aspartyl-proteinase family (33), which we have termed *CdSAP3*. This is the same gene order as observed in the *C. albicans* genome (26). However, comparison of the *C. albicans* and *C. dubliniensis CDR1* ORFs revealed that the *C. dubliniensis* CD36 *CdCDR1* gene contained a TAG translation stop codon at the nucleotide coordinates +2266 to +2268 (codon 756) which corresponded to a single base difference (G to T) that converted the tyrosine (Y756)-encoding TAT codon found in the *C. albicans CDR1* gene to a TAG translation stop signal (Fig. 1). The predicted polypeptide encoded by this shorter ORF was 755 amino acids in length and had a predicted molecular mass of 85 kDa; the *C. albicans* protein is 1,501 amino acids in length and has a predicted molecular mass of 168 kDa. The truncated CdCdr1p was 96.7% identical to the corresponding amino acid sequences of CaCdr1p. In order to confirm that this substitution was not a PCR artifact, this region was sequenced in six clones generated in six separate PCR amplifications and an identical nucleotide sequence was found in each clone. These data strongly suggested that the substitution was present in both alleles of *CdCDR1*.

Identification of polymorphic alleles of *CdCDR1*. In order to determine whether other strains of *C. dubliniensis* harbored the TAG nonsense codon, the region of the *CdCDR1* ORF containing codon 756 was sequenced in *C. dubliniensis* isolates CM1, CM2, CD57, CD51-II, and CD47-IIb (Table 1). In order to achieve this, a 490-bp region of the *CdCDR1* ORF was amplified by PCR with the primer pair 2F-2R (Table 2; Fig. 1). This amplified product encompassed the nucleotide region +2071 to +2557 of the *CdCDR1* ORF, including the TAG codon at the nucleotide coordinates +2266 to +2268 in *C. dubliniensis* strain CD36. Nucleotide sequence analysis of this region demonstrated that *C. dubliniensis* isolates CD51-II and CD47-IIb harbor a nonsense mutation identical to that found in the *CdCDR1* ORF of isolate CD36. However, isolates CD57, CM1, and CM2 contained a tyrosine codon identical to that found in the *C. albicans CDR1* ORF.

The entire nucleotide sequence of the *CdCDR1* gene from *C. dubliniensis* CD57 was determined in order to compare the sequence of a gene without the nonsense mutation with that from strain CD36. The nucleotide sequence of the *C. dubliniensis* CD57 *CdCDR1* ORF was 99.7% identical (containing 9 base differences) to the *CdCDR1* ORF from strain CD36. The predicted amino acid sequence of the CD57 CdCdr1p contained three amino acid substitutions (H60R, V173M, and S1264L) compared to the CD36 sequence. However, the predicted *CdCDR1* ORF from strain CD57 was not interrupted by a premature stop codon at nucleotide positions +2266 to +2268 and was identical in size (4,506 bp) to the homologous *C. albicans CDR1* gene (19). The nucleotide sequence of the *CdCDR1* ORF from strain CD57 was 92% identical to the corresponding sequence of the *C. albicans CDR1* gene. The CD57 *CdCDR1* gene encodes a predicted polypeptide of 169.6 kDa that is 96.5% identical at the amino acid sequence level to the corresponding *C. albicans* sequence. This highly homologous sequence contained the typical features described in *C. albicans* Cdr1p, namely the conserved Walker A and Walker B motifs and an ATP binding motif in the N-terminal hydrophilic domain (19).

The tyrosine-encoding TAT at codon position 756 in isolates CM1, CM2, and CD57 was found to be located within the recognition sequence for the restriction endonuclease *Ssp*I (5′-AATATT). This *Ssp*I recognition sequence was absent in alleles from CD36, CD51-II, and CD47-IIb containing the TAG stop codon (5′-AATAGT). PCR products amplified from isolates CM1, CM2, and CD57 with the primer pair 2F-2R could be digested with the restriction endonuclease *Ssp*I to yield two distinct fragments of 200 and 290 bp, respectively, in agarose gels. However, *Ssp*I digestion of the amplified product from isolates CD36, CD51-II, and CD47-IIb, which contained the TAG nonsense codon, yielded a single band of approximately 500 bp in agarose gels, indicating that the *Ssp*I recognition sequence was absent. We utilized this restriction fragment length polymorphism as the basis of a screening assay to analyze a larger group of *C. dubliniensis* isolates for the presence or absence of the nonsense mutation. A group of 40 *C. dubliniensis* isolates (all isolates in Table 1, excluding derivatives of CD36, CD57, and CD51-II), including representative isolates from the four *C. dubliniensis* genotypes recently described by Gee et al. (8), were analyzed for the presence of the TAT codon by *Ssp*I digestion of PCR products generated from these isolates with the primer pair 2F-2R (Table 1). This analysis revealed that all of the isolates tested ($n = 16$) belonging to *C. dubliniensis* genotypes 2, 3, and 4 harbored *CdCDR1* alleles that contained the TAT tyrosine codon, as *Ssp*I digestion of the 2F-2R-amplified region yielded two fragments in agarose gels (Table 1). However, analysis of 24 genotype 1 *C. dubliniensis* isolates revealed that 14 (58%) harbored *CdCDR1* alleles that could not be digested by *Ssp*I at this region. DNA sequence analysis of the 2F-2R-amplified region from these 14 isolates revealed the presence of a TAG stop codon that disrupted the *Ssp*I recognition sequence (Table 1). These data confirm that the premature stop codon is present in both alleles of *CdCDR1* in these isolates. Two of these isolates were found to harbor *CdCDR1* alleles containing both the TAT and TAG codon sequences, indicating heterozygosity at this locus. In total, 35% (14 of 40) of the *C. dubliniensis* isolates tested were found to contain *CdCDR1* alleles containing the TAG stop codon.

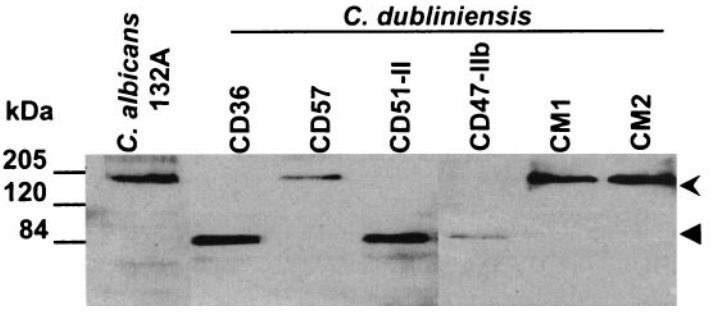

FIG. 2. Western immunoblot analysis of crude protein extracts from *C. albicans* CA132A and several *C. dubliniensis* isolates following SDS-PAGE. Western blots were screened with an anti-Cdr1p polyclonal serum (14). The uppermost arrow at the right of the figure indicates the position of full-length (170 kDa) Cdr1p, which is expressed by *C. albicans* CA132A and by *C. dubliniensis* CD57, CM1, and CM2, respectively. The lower arrow indicates the position of truncated (85 kDa) CdCdr1p, which is expressed by CD36, CD51-II, and CD47-IIb, respectively.

Isolation and sequence analysis of *CdCDR2*. In order to isolate the *C. dubliniensis* homologue of *CDR2*, the primer pair 8F-8R was designed based on the nucleotide sequence of the *C. albicans CDR2* gene (24). This primer pair was used to amplify the complete *C. dubliniensis CdCDR2* gene from the genomic DNA of strain CD36. The amplified *CdCDR2* ORF was 4,503 bp in length, which is 3 bp longer than the *C. albicans CDR2* ORF due to the presence of an additional codon. The *C. dubliniensis CdCDR2* gene shared 91% identity with the *C. albicans CDR2* ORF at the nucleotide sequence level. This ORF was preceded at the 5′ end by a TATA box at position −99 (the first base of the ATG start codon was designated +1). Like *CdCDR1*, the *C. dubliniensis CdCDR2* gene was preceded by a DRE motif at nucleotide coordinates −210 to −189. The *C. dubliniensis CdCDR2* ORF encodes a protein with a predicted molecular mass of 168.9 kDa that is 94.4% identical at the amino acid sequence level to the corresponding *C. albicans* protein. This sequence contained identical Walker A and Walker B motifs and an identical ATP binding motif to that described by Sanglard et al. in the *C. albicans* Cdr2p (24).

Western immunoblot analysis of CdCdr1p. Crude protein extracts of *C. dubliniensis* were analyzed by Western immunoblotting with polyclonal sera raised against the N-terminal region of the *C. albicans* Cdr1p. In protein extracts from *C. albicans* CA132A and *C. dubliniensis* isolates CD41, CD57, CD72, CM1, and CM2 (Table 1), a protein of approximately 170 kDa was detected, matching the predicted size of *C. albicans* Cdr1p and the protein encoded by the full-length *CdCDR1* ORF (Fig. 2). However, in protein extracts from *C. dubliniensis* isolates CD36, CD51-II, CD47-IIb, CO4, and P30, whose *CdCDR1* ORFs were found by sequence analysis to contain a TAG translational stop signal at codon 756, anti-Cdr1p sera reacted with a smaller polypeptide of 85 kDa (Fig. 2). This smaller protein is identical in size to the truncated protein predicted from the *CdCDR1* nucleotide sequence obtained from *C. dubliniensis* strain CD36.

Functional analysis of the *CdCDR1* and *CdCDR2* genes. The *CdCDR1* and *CdCDR2* ORFs were amplified from genomic DNA recovered from *C. dubliniensis* isolates CD36, CD57, CD51-II, CD47-IIb, CM1, and CM2 and *C. albicans* strain CA132A (Table 1). Amplicons were cloned in the *S. cerevisiae GAL1* expression vector pYES (15) and were transformed into the azole-susceptible Δ*pdr5 S. cerevisiae* strain YKKB-13 (24). Transformants were cultured on minimal agar medium containing 2% (wt/vol) galactose as the sole carbon source in order to induce expression of the cloned *CDR* genes. Northern blot analysis of total RNA from galactose-grown transformants revealed high levels of expression of the mRNAs for *CdCDR1* and *CdCDR2* in the respective transformants (data not shown). Transformants were tested for susceptibility to azole antifungal drugs and metabolic inhibitors known to be substrates for *C. albicans* Cdr1p and Cdr2p (19, 24) (Fig. 3). Cloned *CDR1* amplicons from *C. albicans* CA132A and from the *C. dubliniensis* isolates CM1, CM2, and CD57 were found to confer resistance to fluconazole and itraconazole and to the metabolic inhibitors rhodamine 6G, cycloheximide, brefeldin A, crystal violet, and cerulenin in *S. cerevisiae* YKKB-13 (Table 3; Fig. 3A). However, *CdCDR1* amplicons from *C. dubliniensis* isolates CD36, CD51-II, and CD47-IIb, which encoded the truncated CdCdr1p, did not confer resistance to any of these compounds in *S. cerevisiae* YKKB-13 (Fig. 3A), despite the fact that high levels of *CdCDR1* mRNA could be detected in these transformants by Northern analysis (data not shown). For each of these isolates, at least six separate *CdCDR1*-encoding amplicons cloned from separate PCRs were analyzed and identical phenotypes were obtained from each clone.

In order to unequivocally determine if the premature translational stop signal at codon 756 of the *CdCDR1* gene was responsible for the loss of function of the heterologously expressed protein in *S. cerevisiae*, we carried out site-directed mutagenesis to restore the tyrosine-encoding TAT codon at this position in the cloned *C. dubliniensis* CD36 *CdCDR1* gene. Reassignment of this codon fully restored the ability of the expressed protein to impart a drug resistance phenotype in *S. cerevisiae* YKKB-13 compared to that of the truncated *C. dubliniensis* CdCdr1p (Fig. 3B).

The susceptibility of the *CdCDR2*-harboring transformants to fluconazole and itraconazole was also determined. When compared to *S. cerevisiae* harboring the plasmid vector alone (fluconazole MIC, 0.5 μg/ml; itraconazole MIC, <0.03 μg/ml), transformants harboring the *C. albicans CDR2* gene or the *C. dubliniensis CdCDR2* gene were resistant to fluconazole (MIC, 32 to 64 μg/ml) and itraconazole (MIC, 0.5 to 1 μg/ml).

Analysis of *CdCDR1* and *CdCDR2* mRNA expression levels in *C. dubliniensis*. Total RNA was isolated from *C. dubliniensis* isolates and in vitro-generated derivatives with reduced sus-

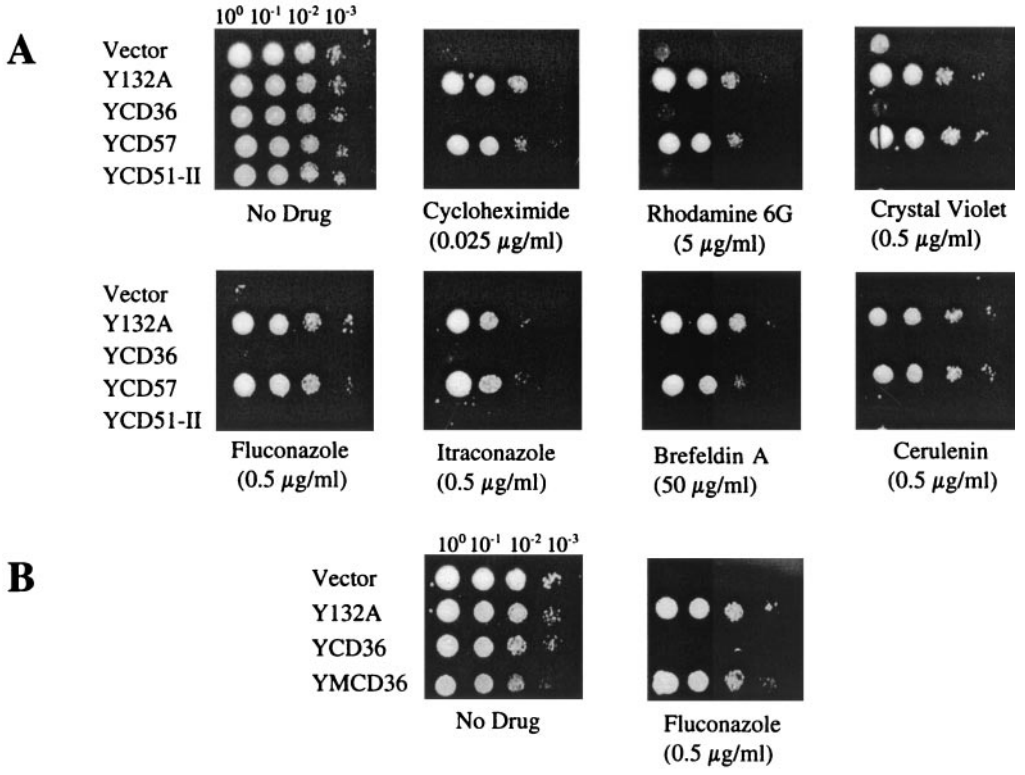

FIG. 3. Susceptibility of *S. cerevisiae* YKKB-13 (*pdr5*) transformants harboring cloned *CDR1* genes to antifungal drugs and metabolic inhibitors. (A) *CDR1* alleles from *C. albicans* and *C. dubliniensis* isolates were amplified by PCR, cloned into the *GAL1* expression vector pYES and transformed into the *S. cerevisiae* strain YKKB-13. The transformants harbored the pYES plasmid (Vector), cloned *CDR1* from *C. albicans* CA132A (Y132A), and cloned *CdCDR1* genes from the *C. dubliniensis* isolates CD36 (YCD36), CD57 (YCD57), and CD51-II (YCD51-II). A suspension (2×10^7 CFU/ml) of each transformant was serially diluted, and 5 μl of each dilution was spotted onto minimal agar medium plates containing fixed concentrations of the antifungal drug or metabolic inhibitor indicated. (B) Fluconazole susceptibility of YKKB-13 transformants harboring the cloned *C. albicans* CA132A *CDR1* gene (Y132A), the CD36 *CdCDR1* gene (YCD36), and the mutated CD36 *CdCDR1* gene (YMCD36) in which the premature stop codon has been reassigned.

ceptibility to fluconazole in order to ascertain the expression levels of *CdCDR1* and *CdCDR2* mRNA in these organisms (Fig. 4). As reported by Moran et al. (13), all clinical isolates and in vitro-generated derivatives of *C. dubliniensis* with reduced susceptibility to fluconazole displayed high levels of *CdMDR1* mRNA compared to matched fluconazole-susceptible isolates (Fig. 4A). *CdMDR1* hybridization signals in the resistant organisms CM2, CD57R, and CD51-II were 50- to 100-fold higher than those observed in matched fluconazole-susceptible isolates. Hybridization of these RNAs with a probe specific for the *CdCDR1* gene (homologous to the region from +1 to +421 of the *CdCDR1* ORF) revealed that several of these isolates and derivatives with reduced susceptibility to fluconazole also displayed comparatively minor increases in levels of *CdCDR1* mRNA expression relative to matched fluconazole-susceptible isolates (Fig. 4B). The oral clinical isolate CM2 (fluconazole MIC, 32 μg/ml) displayed a twofold-higher level of *CdCDR1* mRNA compared to the matched flucon-

TABLE 3. Susceptibility of *S. cerevisiae* YKKB-13 *CdCDR1* transformants to antifungal drugs and metabolic inhibitors determined by broth microdilution

Transformant[a]	MIC (μg/ml) of[b]:						
	FLU	ITRA	CYCL	CRYV	BREF	CER	R6G
Vector (pYES)	0.5	<0.03	0.015	0.25	64	0.06	2
Y132A	64	1	0.5	16	256	2	32
YCD36[c]	0.5	<0.03	0.015	0.25	64	0.06	2
YCD51-II[c]	0.5	<0.03	0.015	0.25	64	0.06	2
YCD57	64	1	0.5	16	256	2	32
YCM1	64	1	0.5	16	256	2	32

[a] Transformants harbor cloned *CDR1* from *C. albicans* CA132A (Y132A), and cloned *CdCDR1* genes from the *C. dubliniensis* isolates CD36 (YCD36), CD51-II (YCD51-II), CD57 (YCD57), and CM1 (YCM1).
[b] FLU, fluconazole; ITRA, itraconazole; CYCL, cycloheximide; CRYV, crystal violet; BRET, brefeldin A; CER, cerulenin; R6G, rhodamine 6G.
[c] The *CdCDR1* genes from *C. dubliniensis* isolates CD36 and CD51-II harbored by these transformants contain a TAG at codon 756.

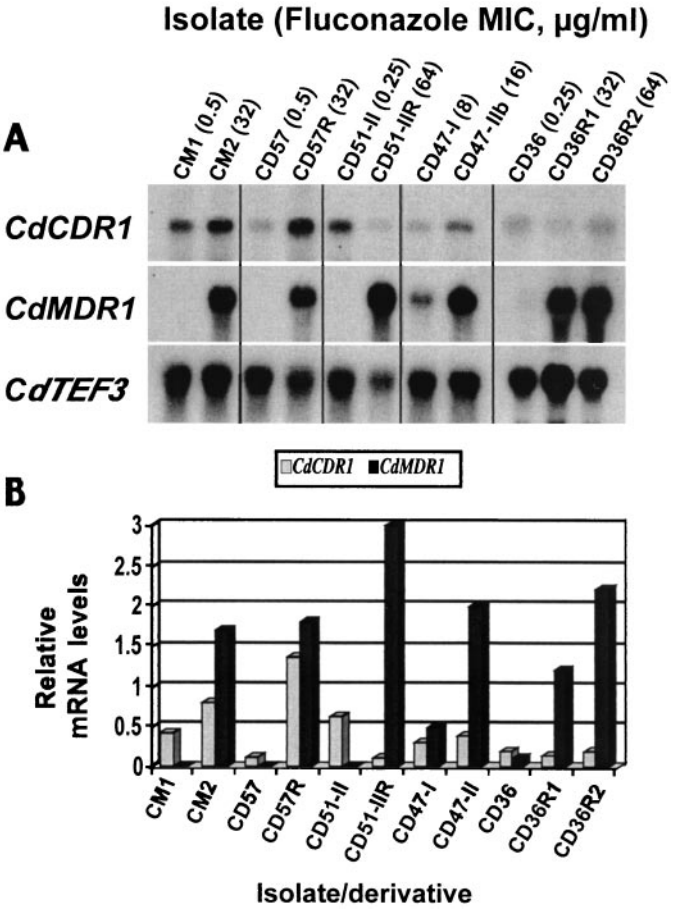

FIG. 4. Northern blot showing expression levels of *CdCDR1*, *CdMDR1*, and *CdTEF3* mRNAs in matched pairs of *C. dubliniensis* clinical isolates and in vitro-generated derivatives exhibiting reduced susceptibility to fluconazole. (A) Total RNA was extracted from *C. dubliniensis* isolates and derivatives grown to the mid-exponential phase in YEPD broth cultures and analyzed by Northern hybridization analysis with [α-^{32}P]dATP-labeled DNA probes homologous to *CdCDR1*, *CdMDR1*, and the constitutively expressed internal control *CdTEF3* gene (see Materials and Methods). (B) Graphical representation of *CdCDR1* and *CdMDR1* mRNA expression levels. Hybridization signals were analyzed by scanning densitometry and normalized against levels of *CdTEF3* expression.

azole-susceptible isolate CM1 (fluconazole MIC, 0.5 μg/ml) recovered from the same patient. Isolates CM1 and CM2 both express the full-length CdCdr1p (Fig. 2). Also, the in vitro-generated derivative CD57R (fluconazole MIC, 32 μg/ml), which also expresses the full-length CdCdr1p, displayed a ninefold-higher level of *CdCDR1* mRNA than the fluconazole-susceptible parental isolate CD57. Interestingly, the oral clinical isolate CD47-IIb (fluconazole MIC, 16 μg/ml), which was found to express the nonfunctional truncated CdCdr1p, expressed a twofold-higher level of *CdCDR1* mRNA than the matched clinical isolate CD47-I (fluconazole MIC, 8 μg/ml). However, in vitro-generated derivatives of the fluconazole-susceptible *C. dubliniensis* isolates CD51-II and CD36 (fluconazole MIC, 0.25 μg/ml), which displayed reduced susceptibility to fluconazole (fluconazole MICs were 32 to 64 μg/ml for CD51-IIR, CD36R1, and CD36R2), displayed levels of *CdCDR1* mRNA that were similar to or reduced in comparison to their respective fluconazole-susceptible parental isolates (Fig. 4B). Both CD36 and CD51-II were shown to express the truncated 85-kDa CdCdr1p (Fig. 2).

Total RNA from these isolates and derivatives was also hybridized with sequences specific for *CdCDR2* (homologous to the region from +1 to +418 of the *CdCDR2* ORF). However, expression of *CdCDR2* mRNA was not detected in any of the isolates or derivatives tested.

Targeted disruption of *CdCDR1*. In order to ascertain the role of the *CdCDR1* gene in determining susceptibility to azole antifungal drugs in *C. dubliniensis*, we disrupted both copies of the *CdCDR1* gene in a *C. dubliniensis* strain by using the *MPA*^{R}-flipper technique described by Wirsching et al. (34, 35). These experiments were carried out in an in vitro-generated derivative of the clinical isolate CD57 (fluconazole MIC, 0.5 μg/ml), termed CD57R, which exhibits reduced susceptibility to fluconazole (fluconazole MIC, 32 μg/ml). CD57R was originally generated by successive subculture of the fluconazole-susceptible isolate CD57 on fluconazole-containing agar medium (13). CD57R expresses the full-length CdCdr1p and showed a ninefold increase in *CdCDR1* mRNA expression levels relative to a matched fluconazole-susceptible isolate (CD57) (Fig. 4B) and also exhibits increased CdCdr1p levels in Western immunoblots (data not shown). In addition, like all *C. dubliniensis* isolates with reduced susceptibility to fluconazole,

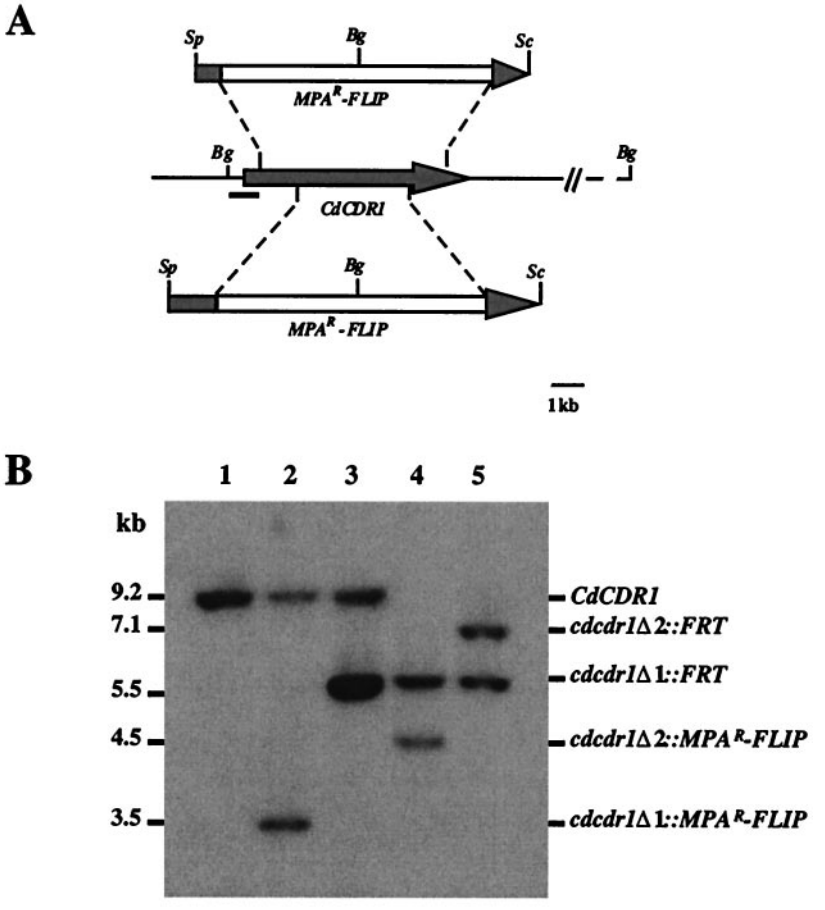

FIG. 5. Inactivation of the *CdCDR1* gene by the *MPA*^{R}-flipper procedure. (A) Restriction map of the *CdCDR1* locus from strain CD57R and allelic replacements with the inserts from pΔCDR1 (top) and pΔCDR2 (bottom). The shaded arrow in the central portion of the figure represents the *CdCDR1* coding region; solid lines represent the *CdCDR1* flanking sequences. The open box represents the *MPA*^{R}-flipper cassette. Restriction endonuclease cleavage sites: Bg, *Bgl*II; Sc, *Sac*I; Sp, *Sph*I. The solid bar represents the region which was used as a probe in Southern hybridization experiments. (B) Southern hybridization of *Bgl*II-digested genomic DNA from *C. dubliniensis* CD57R and mutant derivatives with the 5′ *CdCDR1* probe indicated in panel A. The molecular sizes of the fragments are shown in kilobases to the left of the blot, and the identities of the fragments are shown to the right. Lane 1, CD57R (*CdCDR1*/*CdCDR1*); lane 2, 57RM1 (*CdCDR1*/*cdcdr1*Δ::*MPA*^{R}-*FLIP*); lane 3, 57RM2 (*CdCDR1*/*cdcdr1*Δ::*FRT*); lane 4, 57RM3 (*cdcdr1*Δ::*FRT*/*cdcdr1*Δ::*MPA*^{R}-*FLIP*); lane 5, 57RM4 (*cdcdr1*Δ::*FRT*/*cdcdr1*Δ::*FRT*).

CD57R exhibits an approximately 88-fold increase in expression of *CdMDR1* mRNA (Fig. 4), and this isolate was chosen in order to determine the influence of a *CdCDR1* gene disruption on fluconazole susceptibility in this genetic background. For disruption of the *CdCDR1* gene, a cassette (*MPA*^{R}-flipper) containing the MPA resistance gene and the FLP site-specific recombinase fused to the inducible *C. albicans SAP2* promoter (27), was inserted into the *CdCDR1* ORF to create plasmids pCDRΔ1 and pCDRΔ2, thereby deleting the regions from nucleotide coordinates +538 to +4248 and +1468 to +3532, respectively (Fig. 5A). *C. dubliniensis* CD57R was first transformed with the insert from pCDRΔ1, and MPA-resistant transformants were analyzed by Southern blotting. In strain CD57R (*CdCDR1*/*CdCDR1*), a *Bgl*II DNA fragment of 9.2 kb hybridized with the *CdCDR1* probe (Fig. 5B). Insertion of the fragment from pCDRΔ1 into one of the *CdCDR1* alleles generated a new *Bgl*II fragment of 3.5 kb in strain 57RM1 (*CdCDR1*/*cdcdr1*Δ::*MPA*^{R}-*FLIP*) due to the presence of a *Bgl*II site in the *MPA*^{R}-flipper. Deletion of the cassette by FLP-mediated recombination resulted in the creation of a new 5.5-kb fragment, 3.7 kb smaller than the fragment in the wild-type strain CD57R; this strain was designated 57RM2 (*CdCDR1*/*cdcdr1*Δ::*FRT*). The insert from pCDRΔ2 (Fig. 5A) was then used in a second round of transformation in order to create a deletion in the second *CdCDR1* allele in strain 57RM3 (*cdcdr1*Δ::*FRT*/*cdcdr1*Δ::*MPA*^{R}-*FLIP*). The *MPA*^{R}-flipper was excised again from this transformant to produce a fragment of 7.2 kb in strain 57RM4 (*cdcdr1*Δ::*FRT*/*cdcdr1*Δ::*FRT*).

Disruption of *CdCDR1* affects itraconazole and ketoconazole susceptibility. Heterologous expression of *CdCDR1* in *S. cerevisiae* results in resistance to the azole antifungal drugs fluconazole, itraconazole, and ketoconazole. In order to assess the contribution of the *CdCDR1* gene towards resistance to these agents in *C. dubliniensis*, we compared the azole susceptibility of the *cdr1*-null mutant strain 57RM4 with that of its azole-resistant parental strain CD57R. Broth dilution susceptibility tests were carried out in RPMI 1640 medium against azole drugs for the azole-susceptible clinical isolate CD57, its in vitro-generated fluconazole-resistant derivative CD57R, and the *cdr1* disruptants of CD57R, 57RM2 (*CdCDR1*/

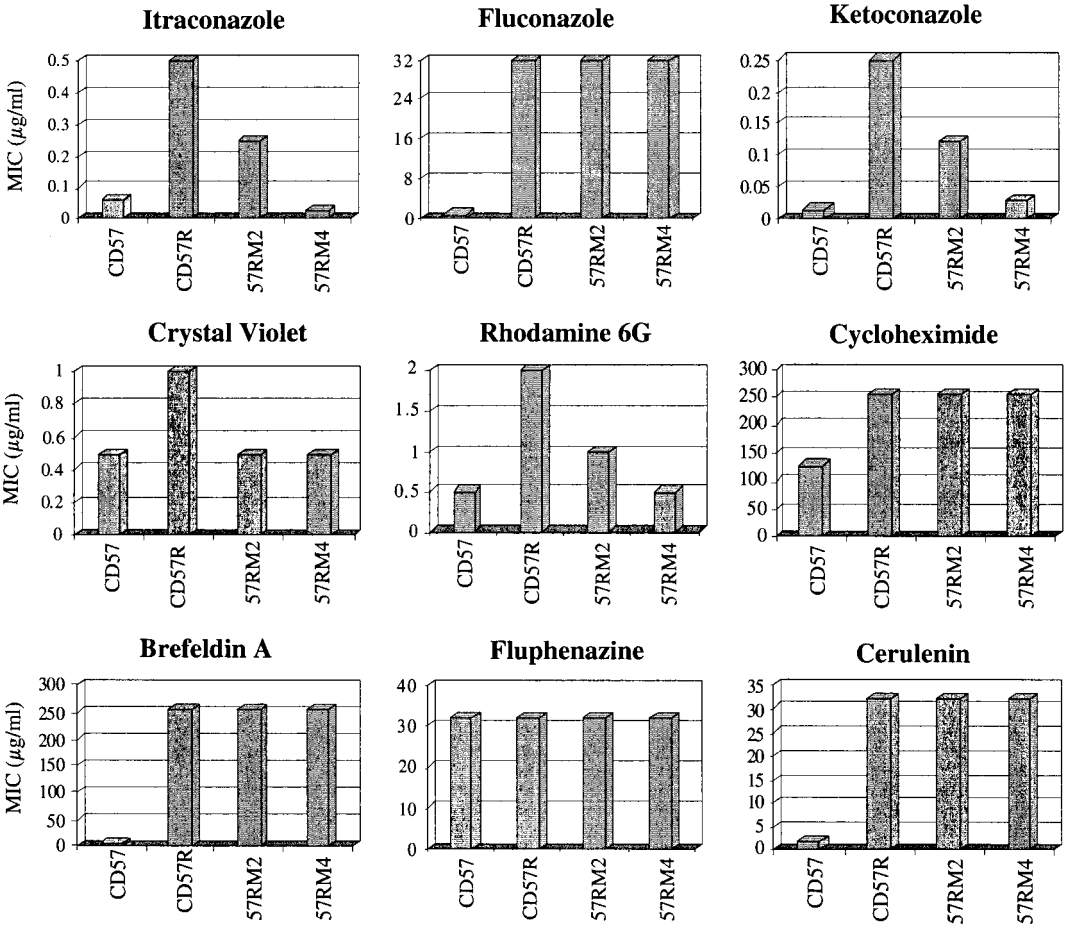

FIG. 6. Susceptibility of *C. dubliniensis* strain CD57, its fluconazole-resistant derivative CD57R, and *CdCDR1* disruptants of CD57R (57RM2 [*CdCDR1*/*cdcdr1*Δ::*FRT*] and 57RM4 [*cdcdr1*Δ::*FRT*/*cdcdr1*Δ::*FRT*]) to fluconazole, itraconazole, ketoconazole, and metabolic inhibitors. MICs were determined by broth microdilution susceptibility testing in RPMI 1640 supplemented with 2% (wt/vol) glucose.

*cdcdr1*Δ::*FRT*) and 57RM4 (*cdcdr1*Δ::*FRT*/*cdcdr1*Δ::*FRT*). Disruption of both copies of *CdCDR1* in 57RM4 resulted in an eightfold increase in susceptibility to both itraconazole (change in MIC from 0.5 to 0.06 μg/ml) and ketoconazole (change in MIC from 0.25 to 0.03 μg/ml) (Fig. 6). The ketoconazole and itraconazole MICs for the double *cdr1* disruptant (57RM4) and CD57, the azole susceptible parental isolate of CD57R, were similar, indicating that expression of the *CdCDR1* gene was responsible for the reduction in susceptibility to these drugs in CD57R. However, this disruption did not affect susceptibility to fluconazole (Fig. 6). As fluconazole, unlike ketoconazole and itraconazole, is also a substrate for the *CdMDR1* multidrug transporter, we hypothesized that the very high levels of expression of the *CdMDR1* transporter in CD57R may mask any effect a deletion of *CdCDR1* may have on fluconazole susceptibility. In order to determine if *CdMDR1* could compensate for the *cdr1* disruption in the presence of other drugs, we analyzed the susceptibility of the *cdr1* null mutant to a range of other metabolic inhibitors. We did not observe any change in the MICs of other drugs that are substrates for both CdCdr1p and CdMdr1p in the *cdr1* disruptant strain 57RM4, including brefeldin A, cerulenin, and cycloheximide (Fig. 6). However, we observed increased susceptibility to crystal violet and rhodamine 6G in strain 57RM4, two drugs that are substrates for CdCdr1p but are not transported by CdMdr1p (Fig. 6). In addition, we did not observe any differences in fluphenazine susceptibility among the isolates and derivatives tested (Fig. 6), indicating that this drug is not a substrate for CdCdr1p.

DISCUSSION

The close relatedness of *C. dubliniensis* to *C. albicans* and the ease with which fluconazole-resistant derivatives of susceptible *C. dubliniensis* clinical isolates can be generated in vitro make *C. dubliniensis* a useful organism to study the development of fluconazole resistance in *Candida* species (13, 14). We and others have isolated *C. dubliniensis* isolates exhibiting fluconazole resistance from the oral cavities of HIV-infected patients, making the investigation of the molecular basis of resistance in this organism worthy of investigation in its own right (10, 14, 20). Recently, molecular genetic techniques, including targeted gene disruption, have been adapted for use in *C. dubliniensis* and will greatly aid in the dissection of this organism's biology (27). Wirsching et al. recently used targeted gene disruption to demonstrate the importance of the major facilitator *CdMdr1p* in the development of fluconazole resistance in *C. dubliniensis* (35). *CdMDR1* mRNA is invariably overexpressed in *C. dubliniensis* strains exhibiting reduced suscepti-

bility to fluconazole, and deletion of this gene in a fluconazole-resistant *C. dubliniensis* isolate rendered the isolate susceptible to fluconazole. In the present study we investigated the role of the second multidrug transporter, CdCdr1p, in fluconazole resistance in *C. dubliniensis*. In *C. albicans*, overexpression of Cdr1p is the most commonly reported mechanism of resistance to azole antifungal drugs (16, 25). Because of this apparent divergence in fluconazole resistance mechanisms in *C. albicans* and *C. dubliniensis*, we decided to investigate in detail the role played by *CdCDR1* in fluconazole resistance in *C. dubliniensis*.

Sequence analysis of the *C. dubliniensis CdCDR1* and *CdCDR2* genes reveals that these genes are highly homologous (>90% identity at the nucleotide sequence level) to their *C. albicans* counterparts (19, 24). The promoter regions of the *C. dubliniensis* genes were also found to contain motifs similar to that of the DRE in *C. albicans* described by de Micheli et al., indicating that expression of these genes may be regulated by similar mechanisms in *C. dubliniensis* (5). However, 35% (14 of 40) of *C. dubliniensis* isolates examined in the present study were found to contain a nonsense mutation approximately midway through the *CdCDR1* ORF. This mutation introduces a premature translation termination signal in the ORF which leads to the translation of a truncated 85-kDa protein (rather than the full-length protein of 169.6 kDa), which we detected in Western immunoblots with an anti-Cdr1p antiserum. We demonstrated that the full-length CdCdr1p and CdCdr2p proteins are functionally equivalent to their *C. albicans* counterparts, as when they are heterologously expressed in a *S. cerevisiae* background they render the host strain less susceptible to a similar range of drugs and metabolic inhibitors. However, expression of *CdCDR1* ORFs containing the nonsense mutation in *S. cerevisiae* did not render the host strain less susceptible to any of the drugs tested, indicating that the truncated CdCdr1p is not capable of mediating a multidrug resistance phenotype. Since the *C. dubliniensis* isolates harboring the nonsense codon containing *CdCDR1* ORFs were obtained from clinical sources, this finding suggests that a fully functional, full-length CdCdr1p is not essential for the normal growth of *C. dubliniensis* in vivo. Furthermore, all of the *C. dubliniensis* isolates containing this nonsense mutation were found to belong to a group of closely related strains recently identified as *C. dubliniensis* genotype 1, as described in the epidemiological study of Gee et al. (8). In the study by Gee et al. (8), four distinct genotypes of *C. dubliniensis* were identified based on DNA fingerprint analysis with the *C. dubliniensis*-specific probe Cd25 and by sequence analysis of the internal transcribed spacer regions of the rRNA genes. Gee et al. found that genotype 1 isolates predominated in an analysis of 98 *C. dubliniensis* isolates. Genotype 1 isolates are mainly recovered from HIV-infected patients, and DNA fingerprint analysis with the *C. dubliniensis*-specific Cd25 probe indicates that they are a closely related group of organisms (mean similarity coefficient value of 0.80 ± 0.06) despite being recovered from disparate geographic areas, indicating that these represent a more recent, and therefore a more homogenous subgroup, of *C. dubliniensis* that has become predominant worldwide (8). The most likely explanation for why the *CdCDR1* TAG mutation is unique to the *C. dubliniensis* genotype 1 population, is that it probably appeared in this subgroup after these organisms separated from the other *C. dubliniensis* genotypes and that it has subsequently spread throughout the human population worldwide. In addition, several of the clinical isolates in which the nonsense mutation was identified in the present study were originally recovered from patients with oral candidiasis who had received fluconazole therapy, indicating that the presence of a truncated CdCdr1p does not appear to adversely affect the ability of these organisms to cause disease. In order to investigate whether the nonsense mutation might have any effect on virulence or on strain selection in patients treated with fluconazole, we propose to conduct growth competition experiments comparing the fitness of strains with and without the *CdCDR1* mutation by using in vivo models with and without fluconazole. Of greater interest is the fact that two of these isolates (CD36 and CD51-II) were capable of yielding fluconazole-resistant derivatives (MIC, 64 μg/ml) upon exposure to fluconazole in vitro, indicating that the full-length CdCdr1p is not essential for the development of fluconazole resistance. Whether the truncated CdCdr1p protein could carry out some alternative cellular function other than drug efflux is as yet unknown.

We have shown that some *C. dubliniensis* isolates and derivatives exhibiting reduced susceptibility to fluconazole that possess the full-length *CdCDR1* ORF exhibit increased transcription of this gene (e.g., CM2 and CD57R) (Fig. 4). We have also observed that fluconazole-resistant derivatives of *C. dubliniensis* isolates possessing the *CdCDR1* ORF containing the nonsense mutation often do not exhibit increases in *CdCDR1* mRNA expression (e.g., CD36R1 and CD36R2) (Fig. 4), and in some cases even exhibit decreased transcription of this gene (e.g., CD51-IIR) (Fig. 4). *CdCDR2* expression has not been detected in vitro in any of the *C. dubliniensis* isolates tested to date, indicating that this gene is stringently repressed.

In order to further investigate the role of functional alleles of *CdCDR1* in the development of fluconazole resistance in *C. dubliniensis*, we carried out targeted disruption of both alleles of this gene in a derivative of the *C. dubliniensis* clinical isolate CD57, termed CD57R, which exhibited reduced susceptibility to fluconazole. As CD57R displays a ninefold-higher level of *CdCDR1* mRNA expression (in addition to overexpression of *CdMDR1*), we anticipated that if *CdCDR1* is significantly involved in the fluconazole resistance phenotype that its disruption would affect susceptibility to all of the azole antifungal drugs, including fluconazole. We found the double *cdr1* disruptant 57RM4 showed increased susceptibility to itraconazole and ketoconazole, and the MICs of these drugs for 57RM4 were similar to those observed for the clinical isolate CD57, the azole-susceptible parent of CD57R, indicating that *CdCDR1* overexpression was the main mediator of reduced susceptibility to these drugs in CD57R (Fig. 6). This phenotype was expected, as both ketoconazole and itraconazole are transported by the heterologously expressed gene in *S. cerevisiae* (Fig. 3). In addition, the double *cdr1* disruptant 57RM4 displayed increased susceptibility to rhodamine 6G and crystal violet (Fig. 6), two metabolic inhibitors which are also transported by CdCdr1p in *S. cerevisiae* (Fig. 3). However, the double *cdr1* disruption did not affect fluconazole susceptibility in 57RM4 when compared to its parental strain CD57R (Fig. 6). However, as the *cdr1* mutant also displayed increased levels of *CdMDR1* mRNA, which encodes a transporter capable of fluconazole efflux, it is perhaps not surprising that fluconazole susceptibility was not affected in this mutant (13). It is likely

that such high levels of *CdMDR1* expression are capable of mediating the fluconazole resistance phenotype alone and may mask the effect of the *cdr1* disruption on fluconazole susceptibility. The *cdr1* mutant was also unaffected in susceptibility to cycloheximide, cerulenin, and brefeldin A (Fig. 3), three compounds which, like fluconazole, are transported by both CdCdr1p and CdMdr1p (13). The findings presented here, which implicate *CdMDR1* gene expression as the most important resistance mechanism to fluconazole and other metabolic inhibitors in *C. dubliniensis* is supported by the findings of Wirsching et al. (35). In that study, Wirsching et al. demonstrated that disruption of the *CdMDR1* gene in the *C. dubliniensis* clinical isolate CM2, which exhibits increased expression of *CdMDR1* mRNA and also exhibits a twofold increase in *CdCDR1* mRNA expression (Fig. 4), was sufficient to render the null mutant susceptible to fluconazole (35). The ketoconazole MICs for the *cdmdr1*-disrupted strain were still elevated, probably due to the elevated *CdCDR1* expression levels seen in this isolate.

In the present study, increased expression of a functional *CdCDR1* gene in the *C. dubliniensis* derivative CD57R mediated an eightfold increase in ketoconazole and itraconazole MICs but did not affect the susceptibility of the isolate to fluconazole (Fig. 6). Previously, many investigators have suggested that multiple efflux mechanisms may contribute to fluconazole resistance in a single isolate (6, 16, 31). It has been suggested that simultaneous activation of multiple fluconazole efflux mechanisms in a single cell would lead to an increased rate of fluconazole efflux and therefore increased resistance to this compound. However, the present study demonstrates that the effects of *MDR1* and *CDR1* overexpression on fluconazole susceptibility in a single strain are not necessarily additive. It is not clear from these data why the Cdr1p and Mdr1p transporters do not have a cumulative effect on fluconazole susceptibility when expressed in concert. Perhaps there is a threshold level of efflux activity, above which the activation of further efflux proteins has no further effect on fluconazole susceptibility. Alternatively, there may be competition between CdCdr1p and CdMdr1p for available substrate (i.e., fluconazole). In this latter scenario, if one transporter has a higher affinity for fluconazole (perhaps in this case CdMdr1p), it may sequester all of the available substrate, rendering transporters with lower affinity for fluconazole redundant. The precise contribution of *CdMDR1* overexpression in fluconazole resistance in specific strains (e.g., CD57RM4 [*cdcdr1Δ::FRT/cdcdr1Δ::FRT*]) will be determined by inactivating the *CdMDR1* gene and determining the effect of this mutation on fluconazole susceptibility.

At present, it is not possible for us to analyze the role of *CdCDR1* as a sole mechanism of fluconazole resistance in *C. dubliniensis* as we have not encountered any *CdCDR1*-overexpressing fluconazole-resistant clinical isolates or in vitro-generated derivatives which do not also coexpress the *CdMDR1* gene to date. In *C. albicans* however, several investigators have described isolates in which *CDR1* is the sole efflux pump activated, indicating that *CDR1*-type efflux pumps are capable of mediating fluconazole resistance in this species (16, 25). However, coactivation of Mdr1p- and Cdr1p-type pumps has been described in fluconazole-resistant isolates of *C. albicans* and *Candida glabrata* and conceivably in these species, as in *C. dubliniensis*, the effects of both pumps on the resistance phenotype may not be additive (6, 16, 23, 25, 31). The data presented here indicate that the roles of individual transporters in the fluconazole resistance phenotypes observed in *C. albicans* and *C. glabrata* species will have to be dissected by targeted disruption of the individual genes in question.

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