Identification and Expression of Multidrug Transporters Responsible for Fluconazole Resistance in *Candida dubliniensis*

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*Candida dubliniensis* is a recently described *Candida* species associated with oral candidosis in human immunodeficiency virus (HIV)-infected and AIDS patients, from whom fluconazole-resistant clinical isolates have been previously recovered. Furthermore, derivatives exhibiting a stable fluconazole-resistant phenotype have been readily generated in vitro from fluconazole-susceptible isolates following exposure to the drug. In this study, fluconazole-resistant isolates accumulated up to 80% less [3H]fluconazole than susceptible isolates and also exhibited reduced susceptibility to the metabolic inhibitors 4-nitroquinoline-N-oxide and methotrexate. These findings suggested that *C. dubliniensis* may encode multidrug transporters similar to those encoded by the *C. albicans* MDR1, CDR1, and CDR2 genes (CaMDR1, CaCDR1, and CaCDR2, respectively). A *C. dubliniensis* homolog of CaMDR1, termed CdMDR1, was cloned; its nucleotide sequence was found to be 92% identical to the corresponding *CaMDR1* sequence, while the predicted CdMDR1 protein was found to be 96% identical to the corresponding *CaMDR1* protein. By PCR, *C. dubliniensis* was also found to encode homologs of CaCDR1 and CDR2, termed CdCDR1 and CdCDR2, respectively. Expression of *CdMDR1* in a fluconazole-susceptible *Apdr5* null mutant of *Saccharomyces cerevisiae* conferred a fluconazole-resistant phenotype and resulted in a 75% decrease in accumulation of [3H]fluconazole. Northern analysis of fluconazole-susceptible and -resistant isolates of *C. dubliniensis* revealed that fluconazole resistance was associated with increased expression of *CdMDR1* mRNA. In contrast, most studies showed that overexpression of *CaCDR1* was associated with fluconazole resistance in *C. albicans*. Increased levels of the CdMdr1p protein were also detected in fluconazole-resistant isolates. Similar results were obtained with fluconazole-resistant derivatives of *C. dubliniensis* generated in vitro, some of which also exhibited increased levels of *CdCDR1* mRNA and CdCdr1p protein. These results demonstrate that *C. dubliniensis* encodes multidrug transporters which mediate fluconazole resistance in clinical isolates and which can be rapidly mobilized, at least in vitro, on exposure to fluconazole.

The triazole antifungal drug fluconazole is commonly used to treat oral candidosis and since its introduction has proved effective in the treatment of oral yeast infections. However, recent studies have reported an increasing incidence of resistance to this compound among clinical isolates of *Candida albicans* from human immunodeficiency virus (HIV)-infected and AIDS patients (14, 15, 25, 31). Furthermore, some evidence suggests that since the introduction of fluconazole, the incidence of infections caused by non-*C. albicans* species of *Candida* has been rapidly generated in vitro from fluconazole-susceptible isolates following exposure to the drug. In this study, fluconazole-resistant isolates accumulated up to 80% less [3H]fluconazole than susceptible isolates and also exhibited reduced susceptibility to the metabolic inhibitors 4-nitroquinoline-N-oxide and methotrexate. These findings suggested that *C. dubliniensis* may encode multidrug transporters similar to those encoded by the *C. albicans* MDR1, CDR1, and CDR2 genes (CaMDR1, CaCDR1, and CaCDR2, respectively). A *C. dubliniensis* homolog of CaMDR1, termed CdMDR1, was cloned; its nucleotide sequence was found to be 92% identical to the corresponding *CaMDR1* sequence, while the predicted CdMDR1 protein was found to be 96% identical to the corresponding *CaMDR1* protein. By PCR, *C. dubliniensis* was also found to encode homologs of CaCDR1 and CDR2, termed CdCDR1 and CdCDR2, respectively. Expression of *CdMDR1* in a fluconazole-susceptible *Apdr5* null mutant of *Saccharomyces cerevisiae* conferred a fluconazole-resistant phenotype and resulted in a 75% decrease in accumulation of [3H]fluconazole. Northern analysis of fluconazole-susceptible and -resistant isolates of *C. dubliniensis* revealed that fluconazole resistance was associated with increased expression of *CdMDR1* mRNA. In contrast, most studies showed that overexpression of *CaCDR1* was associated with fluconazole resistance in *C. albicans*. Increased levels of the CdMdr1p protein were also detected in fluconazole-resistant isolates. Similar results were obtained with fluconazole-resistant derivatives of *C. dubliniensis* generated in vitro, some of which also exhibited increased levels of *CdCDR1* mRNA and CdCdr1p protein. These results demonstrate that *C. dubliniensis* encodes multidrug transporters which mediate fluconazole resistance in clinical isolates and which can be rapidly mobilized, at least in vitro, on exposure to fluconazole.

Clinical isolates tested to date are susceptible to fluconazole (MIC range, 0.125 to 1.0 μg/ml) and to other commonly used antifungal drugs, including ketoconazole, itraconazole, and amphotericin B (9, 17). Based on a limited study, Moran et al. (19) reported the occurrence of fluconazole resistance in 20% of oral isolates (MIC range, 8 to 32 μg/ml) of *C. dubliniensis* recovered from AIDS patients who had been treated previously with fluconazole. Furthermore, sequential exposure of fluconazole-susceptible clinical isolates of *C. dubliniensis* to increasing concentrations of fluconazole in agar medium resulted in the recovery of derivatives which expressed a stable fluconazole-resistant phenotype (MIC range, 16 to 64 μg/ml). It has been suggested that the ability of *C. dubliniensis* to rapidly develop resistance to fluconazole may contribute to its ability to successfully colonize the oral cavities of HIV-infected individuals who are receiving long-term therapy with this compound (19). Furthermore, this may, at least in part, explain the apparent recent emergence of this organism.

Several studies have demonstrated the importance of specific multidrug transporters in the development of fluconazole resistance in *C. albicans*. Sanglard et al. (30, 31) have shown that three *C. albicans* proteins, namely the ATP-binding cassette (ABC) transporters Cdr1p and Cdr2p, encoded by the *CDR1* and *CDR2* genes, respectively, and the major facilitator protein Mdr1p (also known as Benp), encoded by the *MDR1* gene, play important roles in reducing the intracellular flucon-
azole content of fluconazole-resistant *C. albicans* isolates by a process of active drug efflux. White (39) has also demonstrated the importance of these proteins in fluconazole-resistant *C. albicans*. In addition, Löffler et al. (17), Sanglard et al. (29), and White (40) have characterized mutations in the cytochrome P-450 lanosterol 14α-demethylase enzyme, the intracellular target of fluconazole. Some of these mutations have been shown to reduce this protein’s affinity for fluconazole in resistant clinical isolates of *C. albicans* (29, 40).

The objectives of the present study were to investigate the mechanism(s) of fluconazole resistance in *C. dubliniensis* clinical isolates and in in vitro-generated fluconazole-resistant derivatives. Homologs of the *C. albicans* *CDR1, CDR2*, and *MDR1* genes were identified in *C. dubliniensis*. (For maximum clarity, the *C. albicans* and *C. dubliniensis* genes, as well as their products, will be given the prefixes Ca and Cd, respectively.) Expression of these genes was examined in fluconazole-resistant and -susceptible clinical isolates of *C. dubliniensis* in order to characterize their role in fluconazole resistance in this organism.

**MATERIALS AND METHODS**

*C. dubliniensis* clinical isolates, in vitro-generated derivatives, and culture conditions. *C. dubliniensis* isolates were routinely cultured on potato dextrose agar (Oxoid) medium, pH 5.6, at 37°C. For liquid culture, isolates were grown in yeast extract-peptone-dextrose (YPD) broth at 37°C in an orbital incubator. Yeast extract-peptone-dextrose (YPD) broth at 37°C in an orbital incubator. For liquid culture, isolates were grown in YPD broth at 37°C. Isolates were grown in yeast extract-peptone-dextrose (YPD) broth at 37°C in an orbital incubator.

| Isolate or derivative | Source or and comments | MIC (μg/ml) | Reference(s) |
|-----------------------|------------------------|------------|--------------|
| CM1                   | Patient 1              | 0.5 0.03 64 | 1 19 36 |
| CM2                   | Patient 1              | 32 0.12 >256 | 8 19 36 |
| CD48-I                | Patient 2              | 0.5 0.03 32 | 0.5 19 |
| CD48-II               | Patient 2              | 0.5 0.03 32 | 0.5 19 |
| CD47-I                | Patient 4              | 8 0.06 128 | 1 19 |
| CD47-IIa              | Patient 4              | 8 0.12 128 | 1 19 |
| CD47-Iib              | Patient 4              | 16 0.06 128 | 2 19 |
| CD72                  | Patient 16             | 128 0.25 >256 | 2 This study |
| CD51-II               | Patient 8; parental isolate | 0.25 0.015 32 | 0.5 19 |
| CD51-IIA              | Flu derivative of CD51-II | 16 0.015 >256 | 1 19 |
| CD51-IIB              | Flu derivative of CD51-II | 64 0.03 >256 | 4 19 |
| CD51-1IC              | Flu derivative of CD51-II | 64 0.03 >256 | 4 19 |
| CD57                  | Patient 15; parental isolate | 0.5 0.12 32 | 0.5 19 |
| CD57A                 | Flu derivative of CD57 | 0.5 0.06 32 | 0.25 19 |
| CD57B                 | Flu derivative of CD57 | 16 0.12 128 | 1 19 |
| CD57C                 | Flu derivative of CD57 | 32 0.25 >256 | 4 19 |
| CD57D                 | Flu derivative of CD57 | 0.5 0.06 32 | 0.25 19 |
| CD57E                 | Flu derivative of CD57 | 0.5 0.06 32 | 0.25 19 |
| CD57F                 | Flu derivative of CD57 | 8 0.12 128 | 1 19 |
| CD57G                 | Flu derivative of CD57 | 8 0.12 128 | 1 19 |
| CD57H                 | Flu derivative of CD57 | 8 0.12 128 | 1 19 |
| CD57I                 | Flu derivative of CD57 | 32 0.25 >256 | 4 This study |
| CD57J                 | Flu derivative of CD57 | 32 0.25 >256 | 4 This study |
| CD57K                 | Flu derivative of CD57 | 32 0.25 >256 | 4 This study |

*Patient numbers refer to patients in Table 1 of reference 19. *C. dubliniensis* isolate CD72 is first described here (see Materials and Methods). All isolates are oral isolates from HIV-infected or AIDS patients who were previously treated with fluconazole for oral candidosis, with the exception of CD57, which was isolated from a high vaginal swab specimen from an HIV-negative patient. Flu’, fluconazole resistant; Flu”, fluconazole susceptible.

| Reference(s) |
|--------------|
| 19, 36 |
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| 19 |
| 19, 36 |

MICs were determined by the broth microdilution method as described in Materials and Methods.
TABLE 2. Nucleotide sequences of PCR primers used to amplify specific regions of C. dubliniensis DNA

| Primer          | Sequence                  | Nucleotide coordinates | Restriction site(s) | Reference   |
|-----------------|---------------------------|------------------------|---------------------|-------------|
| CDR1F           | 5'-CCGATTCAGGCTGATCTAATTTAG-3' | 25–43                  | BamHI               | 23          |
| CDR1R           | 5'-GGATCCATTTTATTTCTGTC-3'  | 239–257                | BamHI               | 23          |
| CDR2F           | 5'-GGATCCAGGGTGTGAACCTGGAC-3' | 50–66                  | BamHI               | 30          |
| CDR2R           | 5'-GGATCCGCAAGATGGTCAAC-3'  | 160–176                | BamHI               | 30          |
| CmMDR1F         | 5'-AAAACGTTATGCTACAGATTTAGT-3' | 1–22                   | HindIII             | This study  |
| CmMDR1R         | 5'-AAAACGTTATGCTACAGATTTAGT-3' | 1653–1674              | HindIII             | This study  |

* Nucleotide coordinates of the C. albicans or C. dubliniensis gene (where position +1 corresponds to the first base of the ATG translational start codon) on which the nucleotide sequence of the primer is designed. The primer sets CDR1F-CDR1R and CDR2F-CDR2R were designed based on the nucleotide sequence of the C. albicans CDRI and CDR2 genes, respectively (GenBank accession no. X77589 [CDRI] and U68312 [CDRI2]). The CmMDR1F-CmMDR1R primer set was designed based on the nucleotide sequence of the C. dubliniensis CmMDR1 gene determined in this study (Fig. 4).

* Restriction endonuclease recognition sequences included in the primer sequences are underlined.

Accumulation of [3H]fluconazole in C. dubliniensis isolates. Accumulation of [3H]fluconazole (Fluc) in C. dubliniensis and C. albicans was assessed by the method of Sanglard et al. (31). All experiments were repeated on two separate occasions.

DNA isolation and Southern hybridization analysis. Total genomic DNA of C. dubliniensis was isolated from 18 h in YPD broth cultures, as described by Gallagher et al. (6). Large-scale and small-scale E. coli plasmid DNA preparations were as described by Sambrook et al. (32). Restriction endonuclease-digested DNA was transferred to Magna-Gram nylon membranes (MSI, Westboro, Mass.) as described by Sullivan et al. (33), and hybridization reactions were carried out under high-stringency conditions with DNA probes labelled with [α-32P]dATP by random primer labelling in a rotary hybridization oven (Hybaid, Middlesex, United Kingdom) as described by Sullivan et al. (33).

Yeast chromosomes were prepared as described by Vazquez et al. (37) and separated in 1.3% (wt/vol) agarose gels by using the CHEF-Mapper system (Bio-Rad, Hercules, Calif.) as described by Sullivan et al. (36). Chromosome-sized DNA was transferred to nylon membranes for hybridization analysis by standard Southern blotting techniques (33).

Construction of a C. dubliniensis genomic DNA library. High-molecular-mass total cellular DNA from C. dubliniensis CD36 was isolated as described by Bennett et al. (3) and was used for the construction of a lambda EMBL3 library.

Sau3A-generated partial-pit digest products of C. dubliniensis CD36 DNA of greater than 10 kb in size were ligated with BamHI-generated prepared lambda bacteriophage replacement vector EMBL3 arms (Promega) and then packaged in vitro, using the LambdaPack II packaging system (Promega), according to the manufacturer’s instructions. Following packaging, recombinant phage particles were propagated on E. coli P2 lysogenic strain LE392-P2 as described by Sambrook et al. (27). A recombinant phage library containing 2 × 10^10 PFU was obtained from the phage particles.

Recombinant phages were propagated on E. coli LE392 to yield ~600 to 700 PFU per plate for 10-90-mm-diameter petri dishes and were transferred from the plaques onto nitrocellulose membrane filters (Schleicher and Schuell, Dassel, Germany) by overlaying the plaques with the filters. These were then probed by plaque hybridization (27), using as a probe the α-32P-labelled C. albicans MDRI gene containing a 2.9-kb HindIII-BamHI fragment excised from plasmid p2002 (7). The genomic DNA of a recombinant EMBL3 phage, termed 4CD1, which hybridized strongly with the probe was purified as described by Sambrook et al. (27). The cloned DNA insert of 4CD1 was mapped with restriction endonucleases, and specific fragments were subcloned into pBluescript by conventional methods (27).

DNA sequencing. DNA sequencing was performed by the dideoxy chain termination method of Sanger et al. (28), using an Applied Biosystems model 370A automated DNA sequencer. Sequencing reactions were carried out with an Applied Biosystems Prism dye terminator cycle sequencing reaction kit. Searches of the EMBL and GenBank databases for nucleotide and amino acid sequence similarities were performed with the BLAST family of computer programs (2).

PCR amplification of C. dubliniensis DNA sequences. PCR amplification was performed in 100-μ1 reaction volumes containing 100 pmol each of a forward and reverse primer, 10 mM deoxyribonucleoside triphosphates (2.5 mM each), 10 mM MgCl₂, 10 mM Tris (pH 9.0 at 25°C), 50 mM KC1, 0.1% (wt/vol) Triton X-100, 2.5 U of Taq DNA polymerase, and 100 ng of C. dubliniensis genomic DNA. PCR amplification was performed in a DNA thermal cycler (Perkin-Elmer, Norwalk, Conn.). Reactions were carried out with 35 cycles of 1 min for 94°C, primer annealing for 1 min at 55°C, and extension for 2 min at 72°C. Then, the amplified DNA was separated on 1.5% agarose gels and stained with ethidium bromide. The amplified fragments were sequenced using a commercial DNA sequencing service.
gene, the primer set CdMDR1F-CdMDR1R (with HindIII restriction sites at the 5′ ends) (Table 2) was designed based on the nucleotide sequence of the C. dubliniensis CD36 gene determined in this study and the amplification reaction was carried out with a high-fidelity thermostable DNA polymerase (Vent(R) ) from New England BioLabs, Beverly, Mass.). PCR products were cloned into pBluescript II KS(−) by conventional methods (27). Following digestion with HindIII, the CdMDR1 amplicon was cloned into the HindIII-cleaved S. cerevisiae expression vector plasmid pAAH5 to create recombinant plasmid pG3M. Plasmid pAAH5 contains a unique HindIII restriction site downstream of the S. cerevisiae MDR1 promoter, which allows constitutive expression of cloned sequences from this promoter region in S. cerevisiae (31). Both pAAH5 and pG3M were used to transform S. cerevisiae YKBR-13 by standard methods (31).

RNA extraction and Northern analyses. RNA was extracted from C. dubliniensis cultures grown to mid-exponential phase (optical density at 600 nm, 0.6) in 50-ml volumes of YPD broth at 37°C with shaking at 200 rpm in an orbital incubator (Gallenkamp). Extractions were carried out by the glass bead disruption method described by Hube et al. (13). To remove contaminating DNA, 2 volumes of 6 M LiCl was added to each RNA sample, and after incubation of the solutions at −20°C for at least 2 h, they were centrifuged at 11,600 × g. Pelleted RNA was resuspended in diethyl pyrocarbonate-treated water (~150 μl), and 20 μg quantities, in 5- to 10-μl volumes, were used for electrophoresis in 1.2% (wt/vol) agarose gels containing 6% (vol/vol) formaldehyde as described by Hube et al. (13). RNA was transferred onto MagnaGraph nylon membranes by capillary transfer in 20% SSC buffer (3 M NaCl, 0.3 M trisodium citrate). The RNA was fixed by baking the membranes for 30 min at 80°C followed by UV cross-linking in a Bio-Rad UV cross-linker. Hybridization reactions were performed with a 0.7-kb EcoRI–PstI fragment (Table 3) from plasmid pDC1, as described by Hube et al. (13). Relative levels of mRNA expression were measured by using an imaging densitometer (Bio-Rad model GS-670) to scan the hybridization signal intensity on autoradiograms, with the signal intensity of TEF1 mRNA being employed as a loading control.

Results

Susceptibility testing of C. dubliniensis isolates. All of the C. dubliniensis clinical isolates listed in Table 1, except CD72, were tested previously for their susceptibilities to theazole antifungal drugs fluconazole and itraconazole and the polyene antifungal drug amphotericin B (Table 1). The clinical isolate CM2 from patient no. 1 and the CD47 series of isolates from C. dubliniensis, CD47 series N.1 (MIC, 8 to 32 μg/ml) (Table 1). No cross-resistance to itraconazole or amphotericin B was observed for these isolates. The C. dubliniensis clinical isolate CD72, first described in this study, was found to display the highest level of fluconazole resistance (MIC, 128 μg/ml) of all the clinical isolates tested here or previously but was not cross resistant to itraconazole or amphotericin B (Table 1). The fluconazole-resistant C. dubliniensis derivatives previously described by Moran et al. (19) (Table 1) also showed reduced susceptibility to fluconazole compared to their respective parental isolates (MICs, 16 to 64 μg/ml) (Table 1). These derivatives were originally generated by culturing their respective fluconazole-susceptible parental isolates successively on agar media containing increasing concentrations of fluconazole. To investigate the development of fluconazole resistance in C. dubliniensis more closely, the fluconazole-susceptible clinical isolate CD57 was cultured on YPD agar containing progressively increasing concentrations of fluconazole (0.5 to 30 μg/ml). The derivative series generated, CD57C to CD57K (Table 1), followed the development of altered levels of fluconazole susceptibility, from the susceptible parental isolate (CD57) through the final fluconazole-resistant derivative, CD57K. The derivatives CD57C to CD57E were found to exhibit the same fluconazole susceptibility as their parental isolate, CD57 (MIC, 0.5 μg/ml); derivatives CD57F to CD57H each exhibited a fluconazole MIC of 8 μg/ml, and derivatives CD57I-K exhibited a fluconazole MIC of 32 μg/ml. No cross-resistance to itraconazole was exhibited by any of the derivative series CD57C to CD57K (Table 1).

Further susceptibility tests were carried out with methotrexate, 4NQO, cycloheximide, and benomyl. Fluconazole-susceptible isolates and derivatives had methotrexate MICs of 32 to 64 μg/ml, whereas isolates with reduced susceptibility to fluconazole (MICs, ≥8 μg/ml) had MICs which were up to fourfold higher (128 to >256 μg/ml) (Table 1). Similarly, resistance to fluconazole was also associated with reduced susceptibility to itraconazole. Isolates with fluconazole MICs of ≥8 μg/ml had 4NQO MICs which were four- to eightfold higher than those for fluconazole-susceptible isolates and derivatives (Table 1). The MICs of cycloheximide were >256 μg/ml for all of the clinical isolates and derivatives tested, with the exception of the clinical isolates CD48-I and CD48-II, each of which had a cycloheximide MIC of 128 μg/ml, and the fluconazole-susceptible parental isolate CD51-II, which had a cycloheximide MIC of 64 μg/ml. Benomyl MICs were found to range between 16 to 32 μg/ml for all the isolates and derivatives tested. No correlation between fluconazole resistance and increased benomyl or cycloheximide MICs was found. These data suggested that fluconazole resistance in C. dubliniensis is associated with cross-resistance to the structurally unrelated compounds methotrexate and 4NQO and therefore would indicate a multidrug-resistant phenotype.

Accumulation of [3H]fluconazole in fluconazole-susceptible and -resistant cells. To determine if alterations in cellular permeability to fluconazole could be responsible for fluconazole resistance in C. dubliniensis isolates and derivatives, cells were incubated in the presence of [3H]fluconazole and the intracellular contents of this compound in fluconazole-susceptible and -resistant cells were determined. The clinical isolates CM1 (fluconazole MIC, 0.5 μg/ml) and CM2 (fluconazole MIC, 32 μg/ml) were recovered from the same AIDS patient on two separate occasions, and by using DNA fingerprinting it was shown previously that the two isolates were different strains (Table 1) (19). These isolates were exposed to [3H]fluconazole and examined at different time intervals (Fig. 1). The two isolates were found to differ with regard to fluconazole accumulation. The fluconazole-resistant isolate CM2 was found to accumulate 75% less [3H]fluconazole than the susceptible isolate CM1 following a 20-min incubation (Fig. 1). To determine if this was an active, energy-dependent process, both isolates were exposed to a subinhibitory concentration of sodium azide (NaN3; 0.01 mM), which affects the generation of
ATP. NaN₃ had little effect on the fluconazole accumulation level of the fluconazole-susceptible isolate CM1; however, CM2 was found to accumulate approximately three times more fluconazole than CM1 (Fig. 1). Accumulation of [3H]fluconazole in the presence of 0.01 mM NaN₃ was also examined in CM1, CM2, and the fluconazole-resistant in vitro-generated derivatives, including derivatives CD57F to CD57K generated in this study, were also found to accumulate up to 80% less fluconazole than their respective fluconazole-susceptible parental isolates, indicating that a similar mechanism(s) may have been responsible for fluconazole resistance in these derivatives and the clinical isolates described above (Fig. 2B).

**Identification of multidrug resistance genes in C. dubliniensis.** To determine if specific multidrug resistance genes could be responsible for fluconazole resistance in the *C. dubliniensis* clinical isolates and in the in vitro-generated fluconazole-resistant derivatives, it was decided to investigate whether genes encoding multidrug transporters, homologous to those present in *C. albicans*, were present in *C. dubliniensis* (7, 23, 30). Two pairs of oligonucleotide primers, one of which was complimentary to sequences at the 5' end of the *C. albicans* CDR1 gene and the other of which was complimentary to sequences at the 5' end of the CDR2 multidrug resistance genes, were designed (Table 2); the 5' end of these genes were previously shown to contain the largest amount of nucleotide sequence divergence (23, 30). Following PCR amplification with template DNA from *C. dubliniensis* CD36, the CDR1F-CDR1R and CDR2F-CDR2R primer sets in each case yielded single amplimers of approximately 230 and 130 bp, respectively. The nucleotide sequences of the amplimers obtained with the CDR1F-CDR1R and CDR2F-CDR2R primer sets were found to be 91 and 98% identical to the corresponding sequences of the *C. albicans* CDR1 and CDR2 genes, respectively. These findings suggested that *C. dubliniensis* encodes homologs of the *C. albicans* CDR1 and CDR2 multidrug resistance genes, termed CdBDR1 and CdBDR2, respectively.

In an attempt to identify a homolog of the *C. albicans* MDR1 gene in *C. dubliniensis*, a library of *C. dubliniensis* genomic DNA cloned in the lambda replacement vector EMBL3 was screened by plaque hybridization with a radioactively labelled probe consisting of the entire *C. albicans* MDR1 gene. Five reactive plaques were identified, and the phage from the plaque which gave the strongest hybridization signal was chosen for further study and termed phiCD1. Phage phiCD1 was found to contain a cloned DNA insert of approximately 20 kb, coding for approximately 50% less [3H]fluconazole than the susceptible isolates, whereas isolate CD47-Ib from the same patient, with an MIC of 16 µg/ml, accumulated almost 80% less [3H]fluconazole than the susceptible isolates (Fig. 2A). The fluconazole-resistant in vitro-generated derivatives, including derivatives CD57F to CD57K generated in this study, were also found to accumulate up to 80% less fluconazole than their respective fluconazole-susceptible parental isolates, indicating that a similar mechanism(s) may have been responsible for fluconazole resistance in these derivatives and the clinical isolates described above (Fig. 2B).
FIG. 3. Restriction map of CdBMD1-encoding DNA from C. dubliniensis CD36. The black rectangular boxes represent C. dubliniensis genomic DNA. The upper part of the figure shows the 5-kb CdMDRI-encoding EcoRI-XbaI fragment subcloned from recombinant phage 6CD1 into vector plasmid pBluescript, yielding recombinant plasmid pGM1. The lower part of the figure shows the 2.6-kb CdMDRI-encoding SpeI-ClaI fragment of pGM1 subcloned into pBluescript, yielding recombinant plasmid pGM2. The thin double-arrowed line represents the 2-kb fragment of pGM2 insert DNA which was sequenced. The single-arrowed line, indicating the position of and showing the direction of transcription of the 1.674-kb ORF encoding the CdMDRI gene, represents the region which was PCR amplified from pGM2 insert DNA, using a high-fidelity proofreading polymerase, and subcloned into the S. cerevisiae expression vector plasmid pAAh5, yielding recombinant plasmid pGM3. Restriction endonuclease cleavage sites are abbreviated as follows: A, AccI; B, BstI; C, ClaI; E, EcoRI; K, KpnI; R, RsaI; S, SpeI; and X, XbaI.

and Southern hybridization analysis of restriction endonuclease-generated fragments of pCD1 DNA with the C. albicans MDRI gene as a probe identified a strongly hybridizing XbaI-EcoRI insert DNA fragment of 5 kb. This fragment was cloned into pBluescript, and the resulting plasmid was termed pGM1 (Fig. 3). Further restriction endonuclease mapping studies and Southern hybridization analysis with the C. albicans MDRI gene identified a 2.6-kb ClaI-SpeI fragment within the cloned DNA of pGM1; this was also subcloned in pBluescript to yield plasmid pGM2 (Fig. 3). To identify an open reading frame (ORF), approximately 2 kb of the ClaI-SpeI fragment of pGM2 was sequenced on both strands, corresponding to the region between the ClaI site and the RsaI site, as shown in Fig. 3. Computer analysis of the 1,967-bp ClaI-RsaI fragment of pGM2 revealed the presence of one significant ORF of 1,815 bp with two potential ATG start codons at nucleotide positions 141 and +1 (numbering the sequence in the 5′-to-3′ direction from the first base) of the proposed translation start codon [Fig. 4]. The size of the protein encoded by CdBMD1, as determined in Western blotting experiments, and comparison with the corresponding sequence of CaMDRI suggested that the actual coding sequence starts at position +1, as shown in Fig. 4. This proposed start codon is preceded by a putative promoter region in the 5′ flanking sequence, including a CT block at nucleotide positions −104 to −86 and an adenine residue at position −3. Although a number of TA-rich regions were present, none matched the transcription initiation consensus TATAAA (Fig. 4).

This ORF, termed CdBMD1, has the capacity to encode a protein of 557 amino acids with a predicted molecular weight of 62.2 kDa and a pI of 6.4 (Fig. 4). A hydropathy plot generated by the method of Kyte and Doolittle (16) indicates that the structure of the predicted protein encoded by CdBMD1, termed CdMdr1p, is very similar to that of the corresponding C. albicans protein, CaMdr1p, consisting of two halves, each with six putative transmembrane hydrophobic domains, typical of the 12-transmembrane segment (12-TMS) family of drug export proteins within the major facilitator superfamily (MFS) of transporters (11, 21). Also in common with the corresponding C. albicans Mdr1p protein is the presence of a hydrophilic stretch of amino acids near the N terminus.

The C. dubliniensis and C. albicans MDRI genes are highly

FIG. 4. Nucleotide sequence and deduced amino acid sequence of the C. dubliniensis CdBMD1 gene. Nucleotide sequences are numbered in the 5′-to-3′ direction from the first base (+1) of the ATG translation start codon. Amino acid sequences are numbered from the initial methionine. A putative CT block is shown in boldface at nucleotide positions −104 to −86. Amino acid residues which are underlined show the positions of motifs typical of proteins within the MFS of transporter proteins, and residues shown in boldface are those which match the consensus motif as described by Paulsen et al. (21). These correspond to motif D2 (residues 124 to 134), motif A (residues 203 to 215), motif B (residues 249 to 259), and motif G (residues 499 to 505). Also underlined are the WRW and PET motifs, at residues 568 and 570, respectively. The WRW and PET motifs correspond to highly conserved regions in related MFS proteins from S. cerevisiae, although their functions are unknown (11).
homologous, being 92% identical at the nucleotide sequence level, as determined with the CLUSTAL sequence alignment program (12). A number of motifs, described by Paulsen et al. (21), which are conserved within the 12-TMS family of drug export proteins can be identified in the CdMDr1p and CaMDR1p amino acid sequences. Motifs A (CdMDr1p amino acid residues 168 to 180, G x L a D r x G r x x x l, where residues shown in uppercase type are present in at least 70% of aligned sequences analyzed by Paulsen et al. [21] and those shown in lower case are present in approximately 50% of the analyzed sequences) and B (CdMDr1p amino acid residues 203 to 215, l x x x R x x q G g a s) are common throughout the MFS and are believed to play a critical structural role (Fig. 4). Motif A is poorly conserved; however, motif B can be clearly identified in both proteins. Motif C (CdMDr1p amino acid residues 248 to 259, g x x x x G P x x x G x i), which is specific for drug transporters within the MFS, is well conserved and may play a role in drug binding or transport. Motifs D1 (CdMDr1p amino acid residues 124 to 134, l g x x x x P v v x P) and G (CdMDr1p amino acid residues 248 to 259; G x x x G P L) are specific to the 12-TMS transporters and are partly conserved. Also present are the WRW and PET motifs at amino acid residues 265 to 267 and 288 to 290, respectively, as described by Goffeau et al. (11). These motifs, found preceding and just after the sixth transmembrane span, respectively, were identified as highly conserved regions in related MFS proteins from S. cerevisiae, although their function is unknown (11).

Southern hybridization analysis of EcoRI-XhoI restriction endonuclease-digested C. dubliniensis CD36 genomic DNA, with the CdMDR1 gene, localized the CdMDR1 gene to a single 5-kb fragment, identical in size to the fragment isolated from the recombinant phage pCD1 (data not shown). Further analysis of Clal-KpnI-digested CD36 genomic DNA identified a band of approximately 1.4 kb, similar in size to the Clal-KpnI region of pGM2 as shown in Fig. 3, and a band of 5 kb corresponding to the 3′ end of the gene and its flanking sequences. Southern hybridization analysis of chromosome-sized DNA molecules from CD36 separated by pulsed-field gel electrophoresis with the CdMDR1 gene as a probe showed that the CdMDR1 gene was located on a chromosome of approximately 1.3 Mb in size, which is similar in size to C. albicans chromosome no. 6, which has been reported as the chromosomal location of CaMDR1 (7).

Expression of the C. dubliniensis CdMDR1 gene in S. cerevisiae. Sanglard et al. (31) demonstrated that expression of the C. albicans MDR1 gene in an azole-susceptible S. cerevisiae strain led to the expression of a fluconazole-resistant phenotype. In the present study, similar experiments were carried out with the CdMDR1 structural gene, using the S. cerevisiae Δpdr5 mutant strain YKKB-13. The S. cerevisiae PDR5 gene is a functional homolog of the C. albicans CD1 gene, and in S. cerevisiae YKKB-13 the PDR5 deletion renders the organism hypersusceptible to fluconazole. The entire CdMDR1 gene was amplified from CD36, using a high-fidelity thermostable DNA polymerase, with the primer set CdMDR1F-CdMDR1R (Table 2). A single amplification product was obtained, which was cloned into the S. cerevisiae expression vector plasmid pAAH5 via the HindIII restriction endonuclease cleavage sites within the primer sequences, yielding the plasmid pGM3 (Fig. 3). The plasmid pAAH5 contains the promoter for the S. cerevisiae ADC1 gene, which allows for constitutive expression of genes when cloned into this vector in S. cerevisiae. A representative transformant of YKKB-13 harboring pGM3, termed YGM3, was tested for susceptibility to fluconazole and was found to have a fluconazole MIC of 128 μg/ml, whereas the fluconazole MIC for a transformant of YKKB-13 bearing only the vector plasmid pAAH5 (termed YP5) was only 2 μg/ml. No differences in the MICs of itraconazole and ketoconazole were found for YGM3 and YP5. Examination of the fluconazole accumulation levels in YGM3 and YP5 showed that YGM3 accumulated approximately 75% less [3H]fluconazole than YP5. These findings illustrate that CdMDR1 mediated expression of a fluconazole resistance phenotype in S. cerevisiae.

To assess whether CdMDR1 could confer a multidrug resistance phenotype on YGM3, susceptibility to a number of unrelated compounds which are known multidrug transporter substrates was tested. Susceptibility tests were carried out on YPD agar medium as described in Materials and Methods. Transformant YGM3 was found to be less susceptible than YP5 to benomyl, brefeldin A, cerulenin, cycloheximide, fluconazole, 4-NQO, 1,10-phenanthroline, sulfortemuron methyl, and terbinafine (Table 3). This range of substrates is similar to that which has been described for the C. albicans Mdr1p transporter. Reduced susceptibility to amorolfine was also not-

### Table 3. Susceptibility of the S. cerevisiae transformants YP5, harboring vector plasmid pAAH5, and YGM3, harboring pGM3, to metabolic inhibitors

| Inhibitor (concns) | YP5 | YGM3 |
|-------------------|-----|-------|
| None              | 4   | 4     |
| Amorolfine (0.05) | —   | 2     |
| Benomyl (50)      | 0   | 4     |
| Brefeldin A (25)  | 2   | 4     |
| Cerulenin (0.5)   | —   | 4     |
| Cycloheximide (0.025) | 1   | 4     |
| Fluconazole (10)  | —   | 3     |
| 4NQO (0.5)        | —   | 4     |
| 1,10-Phenanthroline (20) | — | 4     |
| Rhodamine 6G (5)  | 0   | 0     |
| Sulfortemuron methyl (50) | 1 | 4     |

* Parenthetical values are concentrations of inhibitor (in micrograms per milliliter) incorporated into YPD agar medium.

* Values refer to the growth on YPD agar of colonies from inocula prepared at various dilutions as described in Materials and Methods. 4, growth at 10^{-4} dilution; 3, growth at 10^{-3} dilution; 2, growth at 10^{-2} dilution; 1, growth at 10^{-1} dilution; 0, growth at 10^{0} (undiluted culture); —, no growth at 10^{-2}, as determined by the method of Sanglard et al. (30).
ed in YGM3, which has not been described previously as a substrate for the *C. albicans* Mdr1p transporter (7, 30).

**Analysis of multidrug resistance gene expression in *C. dubliniensis***. To determine if the fluconazole-resistant clinical isolates and in vitro-generated derivatives of *C. dubliniensis* exhibited increased expression of the *CdCDR1*, *CdCDR2*, or *CdMDR1* gene, Northern blot analysis of total cellular RNA was performed. For analysis of *CdCDR1* and *CdCDR2* expression, the cloned PCR amplimers from *C. dubliniensis* were used as probes. For analysis of *CdMDR1* expression, a 1-kb *AclI* fragment of the *CdMDR1* gene from pGM2 (Fig. 3) was used as a probe. In loading control experiments, RNA was probed with a portion of the *C. albicans* gene encoding translation elongation factor 3 (*TEF3*) (13). *TEF3* signals were detected from all *C. dubliniensis* clinical isolates and derivatives tested. The fluconazole-resistant *C. dubliniensis* derivatives CD57A and CD57B were both found to express increased levels of *CdMDR1* mRNA compared to their fluconazole-susceptible parental isolate, CD57 (Fig. 6). Increased levels of *CdMDR1* expression were also detected in CD57B (Fig. 6). The fluconazole-resistant *C. dubliniensis* derivatives CD51-IIA, CD51-IIIB, and CD51-IIC also overexpressed *CdMDR1* mRNA compared to their fluconazole-susceptible parental isolate, CD51-II (Fig. 6). The derivative series CD57C to CD57K was also examined, and the derivatives CD57C, CD57D, and CD57E, which are fluconazole susceptible (MIC, 0.5 μg/ml), were found to have low or undetectable levels of expression of *CdMDR1* and *CdCDR1* mRNA. In contrast, the fluconazole-resistant derivatives CD57F, CD57G, and CD57H (MIC, 8 μg/ml) were found to express increased levels of *CdMDR1* and *CdCDR1* mRNAs, whereas the fluconazole-resistant derivatives CD57I, CD57J, and CD57K (MIC, 32 μg/ml) were found to express four- to fivefold-higher levels of *CdMDR1* mRNA than derivatives CD57F to CD57H.

Northern analysis of clinical isolates showed that low levels of *CdMDR1* mRNA were detected in the fluconazole-susceptible isolate CM1 (Table 1); however, *CdMDR1* was expressed at approximately 15-fold-higher levels in the fluconazole-resistant isolate CM2, which was recovered from the same patient as CM1 (Fig. 6). CM2 also expressed two times more *CdCDR1* mRNA than CM1. In the fluconazole-susceptible clinical isolates CD48-I and CD48-II, the levels of expression of *CdCDR1* and *CdMDR1* mRNAs were almost undetectable. However, in the CD47 series of isolates from patient no. 4 (Table 1), increased levels of *CdMDR1* mRNAs were observed. CD47-I and CD47-IIa (fluconazole MIC, 8 μg/ml) both showed relatively high-level expression of this gene, while CD47-IIb (fluconazole MIC, 16 μg/ml) expressed a twofold-higher level (Fig. 6). Some expression of *CdCDR1* mRNA was also detected in *C. dubliniensis* isolate CD47-IIb. CD72, with a fluconazole MIC of 128 μg/ml, also expressed higher levels of *CdMDR1* mRNA than the fluconazole-susceptible isolates (Fig. 6). Despite the high fluconazole MIC of CD72, no expression of *CdCDR1* was detected. All of the *C. dubliniensis* clinical isolates and in vitro-generated derivatives were also examined for expression of *CdCDR2* mRNA; however, no signals for this gene were detected.

Expression of the translation products of the *C. dubliniensis* *CdCDR1*, *CdCDR2*, and *CdMDR1* genes in clinical isolates and in vitro-generated derivatives was also investigated by Western immunoblotting. Due to the high degrees of homology between *CaMDR1* and *CdMDR1* and between *CaCDR1* and *CdCDR1* detected in this study it was predicted that rabbit polyclonal antisera raised against the N-terminal fragments of *C. albicans* Mdr1p, Cdr1p, and Cdr2p would recognize the corresponding *C. dubliniensis* proteins. Using these antisera, low levels of CdCdr1p were detected in isolates CD57 and CD57A, which also expressed low levels of *CdCDR1* mRNA. However, increased protein levels were expressed by CD57B, which also expressed higher levels of *CdCDR1* mRNA. In-
terestingly, in the *C. dubliniensis* clinical isolate CD51-II and its fluconazole-resistant derivatives CD51-IIA, CD51-IIB, and CD51-IIC, although CdCDR1 mRNA was detectable, no CdCdr1p was detected by immunoblotting. Increased expression of CdCdr1p was also detected in the in vitro-generated fluconazole-resistant derivatives CD57F, CD57G, CD57H, CD57I, CD57J, and CD57K (Fig. 7), which expressed higher levels of CdCDR1 mRNA than the fluconazole-susceptible derivatives from the same series, including CD57C, CD57D, and CD57E. CdCdr1p expression was also detected in the *C. dubliniensis* clinical isolates. CM2 expressed a twofold-higher level of CdCdr1p than CM1. CD47-I and CD47-IIb both expressed CdCdr1p; however, no CdCdr1p was detected in clinical isolate CD47-IIb from the same patient, despite the observation that noticeable levels of CdCDR1 mRNA were expressed by this isolate.

CdMdr1p expression studies revealed that CdMdr1p was not detected in any of the fluconazole-susceptible isolates or derivatives in which no expression of CdMDR1 mRNA was detected (Fig. 7). However, in fluconazole-resistant clinical isolates and in fluconazole-resistant in vitro-generated derivatives, all of which expressed CdMDR1 mRNA, increased levels of CdMdr1p were detected (Fig. 7). No expression of CdCdr2p was detected by Western immunoblotting in any of the clinical isolates or in vitro-generated derivatives, which is consistent with the absence of CdCdr2 mRNA in these isolates.

**DISCUSSION**

We previously reported the recovery from AIDS patients of oral *C. dubliniensis* isolates with reduced susceptibility to fluconazole and the rapid generation of stable fluconazole-resistant derivatives of them in vitro (19). The purpose of the present study was to investigate the molecular basis of this resistance phenotype in these isolates and derivatives. In addition to confirming their reduced susceptibility to fluconazole, we have also shown that these isolates and derivatives exhibit decreased susceptibility to methotrexate and 4NQO, suggesting a multidrug resistance phenotype. Analysis of [3H]fluconazole accumulation by these organisms indicated that fluconazole-resistant isolates and in vitro-generated derivatives with fluconazole MICs of ≥16 μg/ml accumulated up to 80% less drug than fluconazole-susceptible isolates. Clinical isolates with intermediate levels of fluconazole susceptibility (i.e., an MIC of 8 μg/ml) accumulated approximately 50% less [3H]fluconazole than susceptible isolates, indicating a good correlation between susceptibility and accumulation levels. When accumulation levels were examined in the presence of NaN₃, the fluconazole-resistant isolate CM2 showed a marked increase in the accumulation of [3H]fluconazole, indicating that the fluconazole-resistant phenotype is the result of an active, energy-dependent process. A similar resistance phenotype has been demonstrated to be associated with overexpression of the multidrug transporter genes *CDR1, CDR2*, and *MDR1* in *C. albicans* (1, 30, 31, 39). These findings and the multidrug resistance phenotype exhibited by fluconazole-resistant *C. dubliniensis* prompted us to examine *C. dubliniensis* for the presence of homologs of these genes.

By PCR, we identified sequences in *C. dubliniensis* which are homologous to the *C. albicans* *CDR1* and *CDR2* genes; furthermore, we have cloned a gene from a library of *C. dubliniensis* genomic DNA, termed CdMDR1, which is highly homologous to CaMDR1. The *C. dubliniensis* *MDR1* gene represents the first complete protein-encoding ORF to be determined from this species. Although highly similar to its counterpart in *C. albicans*, CdMDR1 exhibits divergence (8%) at the nucleotide sequence level, which is further supporting evidence that *C. dubliniensis* represents a unique taxon within the genus *Candida* (10, 36). At the amino acid sequence level, CdMdr1p is highly homologous to CaMdr1p, the proteins being 96.2% identical. Most of the differences in amino acid sequence are present in the hydrophilic N-terminal region, which is not thought to form a transmembrane loop in the 12-TMS family of drug export proteins (7, 21). The possible function of this region and its role, if any, in drug efflux have not been examined for CaMdr1p. Both proteins have a predicted topology consistent with that of the 12-TMS family of MFS drug exporters, and a number of amino acid sequence motifs specific for
CD47-IIb, which showed significant levels of CdCDR1 azole-specific azole resistance phenotype when expressed in 4). These findings directly reflect previous findings with the levels of CdCdr1p. All of the raised against the N-terminal regions of CaMdr1p, CaCdr1p, expression in Northern and Western blots with polyclonal sera vitro-generated derivatives was examined by probing for their genes in the fluconazole resistance of clinical isolates and in reduced susceptibility to fluconazole were found to express in- fluconazole MIC of 128 µg/ml and accumulated 75% less [14C]fluconazole than YKKB-13 transformants harboring only the vector (MIC; 2.0 µg/ml). No difference in itraconazole or ketoconazole susceptibility was observed for the two types of transformants. S. cerevisiae harboring pGM3 also exhibited reduced susceptibility to a number of other compounds, including 4NOQ, which suggests that CdMDR1 may be responsible for the multidrug-resistant phenotype of fluconazole-resistant C. dubliniensis isolates and their derivatives.

Involvement of the C. dubliniensis MDR1, CDR1, and CDR2 genes in the fluconazole resistance of clinical isolates and in vitro-generated derivatives was examined by probing for their expression in Northern and Western blots with polyclonal sera raised against the N-terminal regions of CaMdr1p, CaCdr1p, and CaCdr2p. All of the C. dubliniensis clinical isolates with reduced susceptibility to fluconazole were found to express increased levels of CdMDR1 mRNA and CdMdr1p, whereas in fluconazole-susceptible isolates, the levels of expression of CdMDR1 mRNA and CdMdr1p were low or absent (Table 4). These findings directly reflect previous findings with the C. albicans MDR1 gene, which was shown to confer a flucon- azole-specific azole resistance phenotype when expressed in S. cerevisiae (31). Interestingly, CdCdr1p was not detected in CD47-IIb, which showed significant levels of CdCDR1 mRNA.

| Isolate or derivative | Relative (fold) increase in MIC of: | Relative (fold) increase in mRNA of: |
|-----------------------|-------------------------------------|-------------------------------------|
|                       | Fluconazole | Itraconazole | CdMDR1 | CdCDR1 |
| CM2                   | 64         | 4             | 15     | 2      |
| CD47-1b               | 16         | 2             | 9      | 1      |
| CD47-IIa              | 16         | 2             | 10     | 1      |
| CD47-IIb              | 32         | 1             | 25     | 3      |
| CD72a                 | 256        | 4             | 20     | 1      |
| CD51-IIA              | 64         | 1             | 5      | 1      |
| CD51-IIB              | 256        | 2             | 16     | 2      |
| CD51-IIC              | 256        | 2             | 15     | 2      |
| CD57A                 | 32         | 1             | 14     | 1      |
| CD57B                 | 32         | 1             | 17     | 5      |
| CD57C                 | 16         | 2             | 4      | 2      |
| CD57G                 | 16         | 2             | 5      | 2      |
| CD57H                 | 16         | 2             | 4      | 2      |
| CD57I                 | 64         | 4             | 20     | 4      |
| CD57J                 | 64         | 4             | 20     | 4      |
| CD57K                 | 64         | 4             | 25     | 4      |

a Determined by laser densitometry of Northern blot mRNA signals on autoradiograms.
b Baseline fluconazole and itraconazole MICs of 0.5 and 0.06 µg/ml, respectively, were chosen.
c Increased expression of CDR1 mRNA was not correlated with increased levels of CdCdr1p.
atives of *C. dubliniensis* have been found to differ from those of their fluconazole-susceptible parental isolates (19). It is possible that alterations of sequences flanking drug transporter genes or trans-acting factors influence their rates or regulation of transcription (41).

The ability of *C. dubliniensis* to rapidly develop fluconazole resistance in vitro may have implications for antifungal resistance in vivo. If the development of fluconazole resistance in *C. dubliniensis* in vitro correlates with the development of fluconazole resistance in vivo, it may prove to be a useful model system for studying the mechanisms involved in the development of fluconazole resistance in a clinical context. It is still unknown why *C. dubliniensis* has emerged, apparently, only in recent years. However, the appearance of *C. dubliniensis* shortly after the widespread introduction of fluconazole for the treatment of oral candidosis in HIV-infected and AIDS patients, particularly in patients with recurrent infection, may be correlated. Perhaps the ability of *C. dubliniensis* to rapidly switch on expression of the *CdMDR1* gene enables this organism to persist in the oral cavities of patients undergoing fluconazole therapy. However, it is important to note that not all HIV-infected and AIDS patients undergoing fluconazole therapy who are colonized with *C. dubliniensis* yield fluconazole-resistant isolates, a situation similar to that observed with *C. albicans* (15, 24). The development of fluconazole resistance may depend on the dosage of drug administered, the duration of therapy, or the immune status of the patient, as has been observed in the case of *C. albicans* infection (15, 25). In addition, few investigators have examined how *Candida* species respond to fluconazole exposure in vivo, and although we observed stable changes in *C. dubliniensis* multidrug resistance gene expression in vitro, it is not known whether exposure to fluconazole in vivo could lead to changes in multidrug resistance gene expression. Schoofs et al. (32) demonstrated that in vivo populations of *C. albicans* can consist of a large number of subtypes which differ in their relative susceptibility to antifungal agents, and the authors suggested that exposure to fluconazole in vivo could lead to selection of such fluconazole-resistant subtypes. Clearly, in vivo *Candida* populations, including those of *C. dubliniensis*, may have the ability to respond in a dynamic fashion to antifungal therapy, a response which may involve changes in gene expression or the relative abundance of yeast species in the population. To determine the extent and nature of fluconazole resistance in populations of *C. dubliniensis*, epidemiological studies are currently continuing to follow the progress of *C. dubliniensis*-colonized patients who are undergoing fluconazole therapy.

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