Reduced Azole Susceptibility in Genotype 3 *Candida dubliniensis* Isolates Associated with Increased CdCDR1 and CdCDR2 Expression

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*Candida dubliniensis* is a recently identified yeast species primarily associated with oral carriage and infection in individuals infected with the human immunodeficiency virus. The species can be divided into at least four genotypes on the basis of the nucleotide sequence of the internal transcribed spacer region of the rRNA operon. Previous studies have shown that a small number of clinical isolates belonging to genotype 1 are resistant to the commonly used antifungal drug fluconazole. The aim of the present study was to investigate the molecular mechanisms responsible for reduced susceptibility toazole drugs in *C. dubliniensis* genotype 3 isolates obtained from a patient with fluconazole-recalcitrant oral candidiasis. Four isolates from a single clinical sample, one susceptible, the other three exhibiting reduced susceptibilities to fluconazole, itraconazole, ketoconazole, voriconazole, and posaconazole, were examined. Results showed that reduced susceptibility to azole drugs was associated with an increase in the expression of the multidrug transporters CdCDR1 and CdCDR2 which correlated with reduced intracellular accumulation of radiolabeled fluconazole and an increase in the activity of energy-dependent efflux mechanisms. In contrast to observations made in previous studies, overexpression of the multidrug transporter CdmDR1 was not observed. Despite a thorough investigation of all commonly encountered mechanisms ofazole resistance, no other mechanism could be associated with reduced susceptibility to azole drugs in the clinical isolates studied. This is the first report of CdCDR2 involvement inazole resistance in *C. dubliniensis*.

While the introduction of azole antifungal agents has improved the outcome of many fungal infections, treatment failure due to resistance to these agents has become a clinical problem. In particular, resistance to azole drugs has been associated with oral candidiasis relapses in human immunodeficiency virus (HIV)-infected and AIDS patients following exposure to azole drugs (11, 13, 16, 17, 24, 29). Reduced accumulation of the drug due to increased efflux is another mechanism commonly involved inazole resistance in clinical *Candida* isolates (1, 15, 21, 25, 31, 35). In addition, resistance to azole drugs has also been associated with modifications of the ergosterol biosynthetic pathway, such as defects in the sterol C5,6-desaturation step (10, 12, 19, 26).

*Candida dubliniensis*, a recently described species closely related to *Candida albicans*, has been documented as a significant cause of oral disease in HIV-infected patients, particularly those who routinely receive fluconazole therapy for the treatment of oral candidiasis (3, 14, 27, 33). Recently, four distinct genotypes were identified among *C. dubliniensis* isolates on the basis of sequence variations in the ITS1 and ITS2 regions of the rDNA operon (8). Gee et al. found that isolates belonging to genotype 1 were predominant worldwide and were recovered mainly from HIV-infected patients, while isolates belonging to genotypes 2, 3, and 4 were recovered mainly from HIV-negative individuals. Resistance to fluconazole in clinical isolates of *C. dubliniensis* belonging to genotype 1 has been observed previously (22, 25, 28). In addition, we have previously shown that in vitro exposure of *C. dubliniensis* to fluconazole can result in the development of stable resistance (21, 22). Previous molecular studies have shown that fluconazole-specific resistance inazole-resistant *C. dubliniensis* genotype 1 clinical isolates and in vitro-generated derivatives is associated primarily with overexpression of the major facilitator CdmDR1p (21, 39). Although upregulation of CdCDR1 has been observed in fluconazole-resistant clinical isolates and in vitro-generated derivatives (21), it has been shown that CdCDR1p is not essential for fluconazole resistance (20). This is...
in contrast to the findings for C. albicans, where almost all isolates with reduced susceptibility to azoles examined to date show upregulation of CaCDR1 (24). Moreover, Moran et al. found that 58% of C. dubliniensis genotype 1 isolates harbor mutated alleles of CdCdR1 that encode a truncated, nonfunctional CdCdR1p protein.

In the present study, we describe the first case of reduced susceptibility toazole drugs in C. dubliniensis isolates belonging to genotype 3. Using a matched susceptible isolate, an in-depth investigation of all molecular mechanisms previously associated withazole resistance showed that reduced azole susceptibility was associated exclusively with overexpression of CDR (Candida drug resistance) efflux pumps, a mechanism not previously described for genotype 1 C. dubliniensis isolates.

MATERIALS AND METHODS

C. dubliniensis clinical isolates. An AIDS patient showing persistent symptoms of oral candidiasis despite fluconazole therapy attended the Dublin Dental Hospital in November 1997. A single oral swab sample taken from the dorsum of the tongue yielded both C. albicans and C. dubliniensis when plated on CHROMagar Candida (Paris, France) medium (without fluconazole). All 12 single-colony isolates of C. dubliniensis present on the isolation plate were recovered simultaneously from this sample. On the basis of their susceptibilities toazole drugs, four of the clinical C. dubliniensis isolates (one susceptible and three with reduced susceptibility) were selected for detailed molecular analysis. In addition, four single-colony C. albicans isolates were selected and subcultured for further study. The C. dubliniensis isolates were originally identified on the basis of their dark green color on CHROMagar Candida medium, and their identities were confirmed by using phenotypic and molecular techniques, including biotyping and nucleotide sequence analysis of the internal transcribed spacer (ITS) region of the rRNA operon (data not shown). The isolates exhibited a range of susceptibilities to fluconazole (MIC range, 0.25 to 8 μg/ml), itraconazole (MIC range, 0.06 to 0.5 μg/ml), ketoconazole (MIC range, 0.07 to 0.125 μg/ml), voriconazole (MIC range, 0.07 to 0.125 μg/ml), and posaconazole (MIC range, 0.01 to 0.25 μg/ml) (Table 1). In addition, four C. albicans isolates were selected from the initial isolation plate. All four were found to be susceptible to fluconazole (MIC range, 0.25 to 0.5 μg/ml) and itraconazole (MIC, 0.03 μg/ml) (Table 1). Of the 12 C. dubliniensis isolates, 4 representative isolates were selected for further study on the basis of their susceptibilities toazole drugs. One isolate, CD519-8, was susceptible to all theazole drugs tested, while the other three isolates (CD519-1, CD519-7, and CD519-14) showed reduced susceptibility to these drugs (Table 1). Although resistance toazole drugs is frequently associated with resistance to other antifungal drugs and metabolic inhibitors, the four clinical isolates were all susceptible to amphotericin B (MIC, 0.06 μg/ml), and all had similar susceptibilities to 4-nitroquinoline N-oxide, flucytosine, crystal violet, and 1,10-phenanthroline (data not shown). To determine the relatedness between these four isolates, they were analyzed by Southern hybridization with the C. dubliniensis-specific fingerprinting probe Cd25 (9).

The four isolates yielded very similar Cd25-generated fingerprint profiles that were significantly different from the fingerprint profile obtained for the C. dubliniensis type strain, CD36 (AB, 0.24 to 0.26), which was used as a reference in fingerprinting procedures (Fig. 1A). However, the Cd25-generated fingerprint profile of CD519-14 (AB, 0.93) contained three polymorphic bands (Fig. 1A) which were not present in the fingerprint profiles of isolates CD519-1, CD519-7, and CD519-8 (AB, 1.00). This finding suggested that, although isolate CD519-14 was closely related to the other three isolates, it had undergone minor genetic reorganization suggestive of microevolution, which has been observed previously for C. dubliniensis (8, 9). The karyotypes of the four isolates were analyzed by pulsed-field gel electrophoresis. The four clinical isolates yielded identical karyotype profiles which were clearly distinct from the karyotype profile of the C. dubliniensis type strain, CD36, used as a reference (Fig. 1B). Taken together, the results of the Southern blot and karyotype fingerprinting experiments showed that the four isolates examined were clonally related.

In order to elucidate the mechanism(s) responsible for re-

plimers were used directly in sequencing reactions carried out by Lark, Inc. (Saffron Walden, Essex, United Kingdom).

Biochemical analyses. Accumulation of [14]H]fluconazole in C. dubliniensis was assessed by the method of Sanglard et al. (31) using cells grown to mid-log phase in drug-free yeast nitrogen base broth. At a time point of 20 min was used, because this has been shown previously to represent steady-state conditions (21).

For gas chromatography-mass spectrometry (GC-MS), monosaponifiable sterols were extracted and analyzed by using the method described by Pinjon et al. (26).

RESULTS

Twelve single-colony isolates of C. dubliniensis were recovered from the same clinical specimen obtained from a patient with oral candidiasis receiving fluconazole treatment. All were found to belong to C. dubliniensis genotype 3 on the basis of nucleotide sequence analysis of the internal transcribed spacer (ITS) region of the rRNA operon (data not shown). The isolates exhibited a range of susceptibilities to fluconazole (MIC range, 0.25 to 8 μg/ml), itraconazole (MIC range, 0.06 to 0.5 μg/ml), ketoconazole (MIC range, 0.07 to 0.125 μg/ml), voriconazole (MIC range, 0.07 to 0.125 μg/ml), and posaconazole (MIC range, 0.01 to 0.25 μg/ml) (Table 1). In addition, four C. albicans isolates were selected from the initial isolation plate. All four were found to be susceptible to fluconazole (MIC range, 0.25 to 0.5 μg/ml) and itraconazole (MIC, 0.03 μg/ml) (Table 1). Of the 12 C. dubliniensis isolates, 4 representative isolates were selected for further study on the basis of their susceptibilities toazole drugs. One isolate, CD519-8, was susceptible to all theazole drugs tested, while the other three isolates (CD519-1, CD519-7, and CD519-14) showed reduced susceptibility to these drugs (Table 1). Although resistance toazole drugs is frequently associated with resistance to other antifungal drugs and metabolic inhibitors, the four clinical isolates were all susceptible to amphotericin B (MIC, 0.06 μg/ml), and all had similar susceptibilities to 4-nitroquinoline N-oxide, flucytosine, crystal violet, and 1,10-phenanthroline (data not shown). To determine the relatedness between these four isolates, they were analyzed by Southern hybridization with the C. dubliniensis-specific fingerprinting probe Cd25 (9).

The four isolates yielded very similar Cd25-generated fingerprint profiles that were significantly different from the fingerprint profile obtained for the C. dubliniensis type strain, CD36 (SAB, 0.24 to 0.26), which was used as a reference in fingerprinting procedures (Fig. 1A). However, the Cd25-generated fingerprint profile of CD519-14 (SAB, 0.93) contained three polymorphic bands (Fig. 1A) which were not present in the fingerprint profiles of isolates CD519-1, CD519-7, and CD519-8 (SAB, 1.00). This finding suggested that, although isolate CD519-14 was closely related to the other three isolates, it had undergone minor genetic reorganization suggestive of microevolution, which has been observed previously for C. dubliniensis (8, 9). The karyotypes of the four isolates were analyzed by pulsed-field gel electrophoresis. The four clinical isolates yielded identical karyotype profiles which were clearly distinct from the karyotype profile of the C. dubliniensis type strain, CD36, used as a reference (Fig. 1B). Taken together, the results of the Southern blot and karyotype fingerprinting experiments showed that the four isolates examined were clonally related.

In order to elucidate the mechanism(s) responsible for re-
duced susceptibility to azole drugs in these isolates, we carried out a thorough analysis investigating the mechanisms commonly associated with azole resistance in *C. dubliniensis* (20, 21, 25, 26).

We have recently shown that resistance to azole drugs in *C. dubliniensis* can be associated with defective sterol C5,6-desaturation, resulting in a lack of ergosterol synthesis and the accumulation of atypical sterol precursors (26). In order to determine whether the decreased susceptibilities to azole drugs observed in clinical isolates CD519-1, CD519-7, and CD519-8 could be due to a defect in the enzyme sterol C5,6-desaturase, the nonsaponifiable sterols present in their membranes were analyzed by GC-MS. This analysis showed that all four clinical isolates accumulated ergosterol in their membranes, which indicated that the ergosterol synthesis pathway, and more particularly the sterol C5,6-desaturation function, was intact in all four clinical isolates (Table 2).

In order to investigate whether overexpression of the enzyme lanosterol demethylase (*CdErg11p*) was involved in me-

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**TABLE 1. Susceptibilities to azole drugs of the *C. dubliniensis* and *C. albicans* isolates recovered from the same clinical sample**

<table>
<thead>
<tr>
<th>Isolate</th>
<th>Fluconazole (µg/ml)</th>
<th>Itraconazole (µg/ml)</th>
<th>Ketoconazole (µg/ml)</th>
<th>Voriconazole (µg/ml)</th>
<th>Posaconazole (µg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>C. dubliniensis</em></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CD519-1</td>
<td>8</td>
<td>0.5</td>
<td>0.125</td>
<td>0.125</td>
<td>0.25</td>
</tr>
<tr>
<td>CD519-2</td>
<td>4</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>CD519-3</td>
<td>4</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>CD519-4</td>
<td>0.25</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>CD519-5</td>
<td>4</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>CD519-6</td>
<td>8</td>
<td>0.125</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>CD519-7</td>
<td>8</td>
<td>0.25</td>
<td>0.125</td>
<td>0.125</td>
<td>0.125</td>
</tr>
<tr>
<td>CD519-8</td>
<td>0.5</td>
<td>0.06</td>
<td>&lt;0.07</td>
<td>0.07</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>CD519-10</td>
<td>4</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>CD519-11</td>
<td>2</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>CD519-13</td>
<td>4</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>CD519-14</td>
<td>0.25</td>
<td>0.03</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td><em>C. albicans</em></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CA1197-1</td>
<td>0.25</td>
<td>0.03</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>CA1197-2</td>
<td>0.25</td>
<td>0.03</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>CA1197-3</td>
<td>0.25</td>
<td>0.03</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>CA1197-4</td>
<td>0.5</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
</tbody>
</table>

*a* Boldfaced isolates were selected for more-detailed phenotypic and molecular analysis.

*b* MICs were determined by BMD as described in Materials and Methods. ND, not determined.

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**TABLE 2. Sterols accumulated by *C. dubliniensis* clinical isolates in order of retention time**

<table>
<thead>
<tr>
<th>Isolate</th>
<th>Accumulated sterols (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>CD519-1</td>
<td>Ergosta-tetraenol (15.3)</td>
</tr>
<tr>
<td></td>
<td>Ergosterol (61.2)</td>
</tr>
<tr>
<td></td>
<td>Episterol (8.3)</td>
</tr>
<tr>
<td></td>
<td>Obtusifoliol (2.8)</td>
</tr>
<tr>
<td></td>
<td>Unknown sterols (12.4)</td>
</tr>
<tr>
<td>CD519-7</td>
<td>Ergosta-tetraenol (17.8)</td>
</tr>
<tr>
<td></td>
<td>Ergosterol (61.5)</td>
</tr>
<tr>
<td></td>
<td>Fecosterol (3.7)</td>
</tr>
<tr>
<td></td>
<td>Episterol (7.5)</td>
</tr>
<tr>
<td></td>
<td>Obtusifoliol (3.9)</td>
</tr>
<tr>
<td></td>
<td>Unknown sterols (6.7)</td>
</tr>
<tr>
<td>CD519-8</td>
<td>Ergosta-tetraenol (16.8)</td>
</tr>
<tr>
<td></td>
<td>Ergosterol (55.8)</td>
</tr>
<tr>
<td></td>
<td>Fecosterol (6.7)</td>
</tr>
<tr>
<td></td>
<td>Episterol (6.0)</td>
</tr>
<tr>
<td></td>
<td>Obtusifoliol (1.8)</td>
</tr>
<tr>
<td></td>
<td>Unknown sterols (12.8)</td>
</tr>
<tr>
<td>CD519-14</td>
<td>Ergosta-tetraenol (21.3)</td>
</tr>
<tr>
<td></td>
<td>Ergosterol (63.2)</td>
</tr>
<tr>
<td></td>
<td>Fecosterol (2.9)</td>
</tr>
<tr>
<td></td>
<td>Episterol (5.0)</td>
</tr>
<tr>
<td></td>
<td>Obtusifoliol (3.1)</td>
</tr>
<tr>
<td></td>
<td>Unknown sterols (4.5)</td>
</tr>
</tbody>
</table>

*a* Sterols were extracted from cells grown overnight in YEPD broth at 30°C and were analyzed by GC-MS as described in Materials and Methods.
There was no significant elevation of Cd 
ERG11 
ical isolate CD519-8 were examined by Northern blot analysis. 
in any of the four clinical isolates examined (Fig. 2).
lished Cd 
ERG11 
erozygous polymorphism (V402G) relative to the recently pub-
homozygous polymorphisms (I188V and R499K) and one het-
ERG11 
identical Cd 
amplified by PCR and sequenced directly. All four isolates had 
C. dubliniensis 
CD519-1, CD519-7, and CD519-14, expression levels of the 
diating reduced susceptibility toazole drugs in clinical isolates 
C. dubliniensis 
CDR2, C. dubliniensis 
CDR1, C. dubliniensis 
TEF3 gene. Expression of C. dubliniensis 
MRR1, C. dubliniensis 
MDR1, C. dubliniensis 
MDR1 gene. Expression of C. dubliniensis 
TEF3 was used as an internal control for RNA loading.
diating reduced susceptibility toazole drugs in clinical isolates 
C. dubliniensis 
CD519-1, CD519-7, and CD519-14, expression levels of the 
C. dubliniensis 
ERG11 gene in these isolates and the azole-susceptible clinical 
isolate CD519-8 were examined by Northern blot analysis. 
there was no significant elevation of Cd 
ERG11 mRNA levels in any of the four clinical isolates examined (Fig. 2).
In addition, the Cd 
ERG11 genes from all four isolates were amplified by PCR and sequenced directly. All four isolates had 
C. dubliniensis 
ERG11 nucleotide sequences which contained two homozygous polymorphisms (I188V and R499K) and one het-
erozygous polymorphism (V402G) relative to the recently published C. dubliniensis 
ERG11 sequence (25). To confirm this, the 
C. dubliniensis 
ERG11 genes from two isolates with differing suscep-
C. dubliniensis 
tibilities toazole drugs (i.e., CD519-1 and CD519-8) were expressed in the azole-hypersusceptible Saccharomycyes cerevisiae 
strain YKKB-13 as described by Sanglard et al. (29). The 
susceptibilities toitraconazole and fluconazole of the S. cerevi-
siae transformants expressing C. dubliniensis 
ERG11 genes from clinical isolates CD519-8 and CD519-1 were determined by BMD. 
there was no difference in susceptibility toazole drugs be-
tween transformants expressing the C. dubliniensis 
ERG11 gene from the azole-susceptible isolate CD519-8 and transformants expressing the C. dubliniensis 
ERG11 gene from the isolate with reduced susceptibility toazoles, CD519-1 (data not shown).
In order to determine if reduced accumulation of drug contributed to the difference in MICs observed between the 
C. dubliniensis 
CD519 clinical isolates, levels of [3H]fluconazole accumulation in all four isolates were examined. The isolates and the type 
strain, CD36, were examined at a single time point following a 
20-min exposure to [3H]fluconazole (Fig. 3). The control 
strain, CD36, which harbors a defective Cd 
ERG11 gene (20), accumulated 518 ± 0.7 cpm/10^7 cells. The three isolates with reduced susceptibility toazoles (CD519-1, CD519-7, and CD519-14) showed lower levels of [3H]fluconazole accumulation than the azole-susceptible isolate CD519-8. The azole-
susceptible isolate CD519-8 yielded an average of 471 ± 29 
CPM/10^7 cells following a 20-min exposure to [3H]fluconazole, while isolates CD519-1, CD519-7, and CD519-14, which had 
reduced susceptibilities toazoles, yielded averages of 253 ± 3, 
272 ± 30, and 308 ± 57 cpm/10^7 cells, respectively. This 
showed that in these three isolates, reduced susceptibility toazoles was associated with reduced intracellular accumulation 
of drug, suggesting an increased efflux of fluconazole in these isolates.
In order to more closely examine energy-dependent efflux mechanisms, glucose-mediated efflux of rhodamine 6G was 
measured in the clinical isolates with reduced susceptibility toazole drugs. The method used in the present study directly 
assessed the efflux of R6G by measuring extracellular R6G 
concentrations following the addition of glucose to energy-
starved cells in the absence of fluconazole. Efflux of R6G from 
C. dubliniensis 
CD36 clinical isolates with reduced susceptibility toazole drugs (CD519-1, CD519-7, and CD519-14) and in a clonally related azole-susceptible isolate 
(CD519-8). Accumulation levels were determined following a 20-min 
incubation in the presence of [3H]fluconazole. The C. dubliniensis 
type strain CD36 was used as a control in this experiment.
**FIG. 5.** Susceptibilities of *S. cerevisiae* YKKB-13 (Δpdr5) transformants harboring cloned *CdMDR1* genes to fluconazole and metabolic inhibitors. *CdMDR1* alleles from *C. dubliniensis* isolates were amplified by PCR, cloned into the pAAH5 expression vector (2), and transformed into the *S. cerevisiae* strain YKKB-13. The transformants harbor the pAAH5 plasmid alone (YP5) or pAAH5 carrying the *CdMDR1* gene from isolate CD36 (YGM3) or CD519-1 (EPY84). Each transformant was grown to the exponential-growth phase (density, $2 \times 10^7$ cells/ml), and 4 µl was spotted in a dilution series onto minimal agar medium plates containing fixed concentrations of fluconazole or metabolic inhibitors as indicated. Plates were incubated for 48 h at 30°C.

*C. dubliniensis* type strain CD36, harboring a nonfunctional *CdCDR1* gene, was lower than R6G efflux from the clinical isolates (Fig. 4). In the absence of glucose, the extracellular R6G concentrations were similar in all four isolates, reflecting similar levels of R6G uptake (data not shown). However, in the presence of glucose, R6G efflux from the azole-susceptible isolate CD519-8 was lower than that from isolates CD519-1, CD519-7, and CD519-14 (Fig. 4). This showed an increase in energy-dependent efflux in the three isolates with reduced susceptibility to azole drugs.

All *C. dubliniensis* isolates with a defective *CdCDR1* gene identified to date belong to genotype 1 (17). Therefore, since the CD519 isolates belong to *C. dubliniensis* genotype 3, they were expected to harbor a functional *CdCDR1* gene. In order to investigate the functionality of this gene, PCR amplification followed by restriction fragment length polymorphism analysis was carried out. Moran et al. have shown that the presence of a stop codon at position 756 of the *CdCDR1* ORF removes a restriction site for the enzyme SspI (20). As expected, none of the CD519 isolates harbored the stop codon at position 756 of the *CdCDR1* ORF. Northern blot analysis was carried out in order to determine if the observed increase in energy-dependent efflux in the clinical isolates with reduced susceptibility to azole drugs correlated with increased expression of multidrug resistance genes. Isolates CD519-1, CD519-7, and CD519-14, with reduced susceptibility to azole drugs, showed higher mRNA levels of the two multidrug resistance genes *CdMDR1* and *CdCDR2* than the azole-susceptible isolate CD519-8. However, this was not the case for the multidrug resistance gene *CdMDR1*, expression of which was not detectable in any of the four clinical isolates (Fig. 2). *CdMDR1* expression was increased approximately five-, two-, and sixfold in isolates CD519-1, CD519-7, and CD519-14, respectively. Expression of *CdCDR2* was increased >10-fold in isolates CD519-1, CD519-7, and CD519-14. Since RNA was extracted from cultures grown in the absence of fluconazole, it can be assumed that upregulation of *CdCDR1* and *CdCDR2* is constitutive.

In contrast to previously established mechanisms of resistance to fluconazole in *C. dubliniensis*, reduced susceptibility to azole drugs in *C. dubliniensis* clinical isolates CD519-1, CD519-7, and CD519-14 did not correlate with overexpression of *CdMDR1*. For this reason, it was decided to study the *CdMDR1* gene from the four *C. dubliniensis* CD519 isolates. The ORF and promoter sequences from these isolates were amplified by PCR using primer pairs CdMDR1F-CdMDR1R and PROF-PROR, respectively, and were sequenced. Fragments of the expected sizes (approximately 1.7 kb for the *CdMDR1* ORF and 920 bp for the *CdMDR1* promoter) were obtained in each case. Sequence analysis of the four ORFs showed that the sequences obtained for the four clinical isolates were identical and contained five polymorphisms (D32G, T68S, A105, T307I, and E415K) which affected the amino acid sequence of the *CdMdr1p* protein relative to the published sequence of *CdMDR1* obtained from the *C. dubliniensis* type strain CD36 (EMBL accession no. AJ227752) (21).

Sequence analysis of the *CdMDR1* promoter region showed that promoter sequences obtained from clinical isolates CD519-1, CD519-7, CD519-8, and CD519-14 were identical. However, there were 13 nucleotide differences between the sequence previously obtained from the type strain, CD36, and the sequence data obtained from the four isolates CD519-1, CD519-7, CD519-8, and CD519-14. Finally, in order to determine if the *CdMDR1* gene from the CD519 clinical isolates was functional, it was heterologously expressed in the azole-hypersusceptible *S. cerevisiae* strain YKKB-13. The transformant expressing the *CdMDR1* gene from isolate CD519-1 exhibited a susceptibility pattern identical to that of the transformant expressing the *CdMDR1* gene from the *C. dubliniensis* type strain, CD36. Both transformants showed lower susceptibilities to fluconazole, benomyl, cycloheximide, and 1,10-phenanthroline than the transformant harboring the empty plasmid vector (Fig. 5).

**DISCUSSION**

It is now well established that long-term treatment of *Candida* infections with azole drugs can result in the development of antifungal resistance. In particular, fluconazole treatment of oral candidiasis in HIV-infected and AIDS patients has been associated with treatment failures (23, 36, 37). The recently described species *C. dubliniensis* has been shown to be particularly prevalent in this patient cohort, and the recovery of *C. dubliniensis* genotype 1 isolates exhibiting reduced susceptibility or resistance to fluconazole has been described previously (22, 25, 28). The present study concentrated on the investigation of the molecular mechanisms involved in mediating reduced susceptibility to azoles in four clonally related *C. dub-
C. dubliniensis genotype 3 oral isolates recovered from an AIDS patient with recurrent oral candidiasis recalcitrant to fluconazole treatment. One isolate (CD519-8) was susceptible to azole drugs, while the other three (CD519-1, CD519-7, and CD519-14) showed decreased susceptibilities to the azole drugs fluconazole, itraconazole, ketoconazole, voriconazole, and posaconazole (Table 1).

The decreased susceptibility to azole drugs of the three isolates was not associated with overexpression or mutation of the CdERG11 gene (Fig. 2). There was no indication of membrane permeability alterations in these isolates. Indeed, all four isolates accumulated ergosterol in their membranes (Table 2), suggesting that defects in the enzyme sterol C5,6-desaturase were not involved in mediating reduced susceptibility to azole drugs in these clinical isolates.

Reduced susceptibility to azole drugs was, however, associated with reduced intracellular fluconazole accumulation (Fig. 3). This association was confirmed by Northern blot analysis, which showed a correlation between overexpression of the multidrug resistance genes CdCDR1 and CdCDR2 and a reduction in azole susceptibility (Fig. 2). In contrast to previous studies of fluconazole resistance mechanisms in C. dubliniensis, overexpression of the multidrug resistance gene CdMDR1 was not observed in the three clinical isolates exhibiting reduced susceptibility to azole drugs. Although resistance to fluconazole in C. dubliniensis clinical isolates has been associated with combinations of different molecular mechanisms (25), the primary mechanism of resistance to fluconazole in this species has been shown previously to be due to reduced intracellular drug accumulation mediated by the overexpression of CdMDR1.

While overexpression of CDR2 has been observed in azole-resistant isolates of C. albicans (30), overexpression of its C. dubliniensis homologue, CdCDR2, had never been reported previously and was therefore thought not to be implicated in azole drug resistance in this species. However, heterologous expression of CdCDR2 in S. cerevisiae has previously shown that it is able to mediate resistance to fluconazole and itraconazole (20). Because both CdCDR1 and CdCDR2 were found to be upregulated in the present study, it is not possible to establish the exact contribution of CdCDR2 overexpression to the phenotype. In order to investigate this, it would be necessary to disrupt the CdCDR2 and/or the CdCDR1 gene.

Although five amino acid-altering polymorphisms (D32G, T68S, A105, T307L, and E415K) were identified in the sequence of the CdMDR1 ORF obtained from the four CD519 isolates, heterologous expression in S. cerevisiae YKK813 showed that the CdMDR1 gene was functional, since it was able to mediate resistance to fluconazole and metabolic inhibitors with the same efficiency as the CdMDR1 gene from the C. dubliniensis type strain, CD36 (Fig. 5). While several polymorphisms were identified in the promoter sequences of the CdMDR1 genes from the CD519 clinical isolates relative to the promoter sequence obtained from the C. dubliniensis type strain, CD36 (EMBL accession no. AJ227752), it is not known whether these could affect the expression of CdMDR1 in these isolates (38). A comparative functional analysis of the CdMDR1 promoter from the CD519 isolates and the C. dubliniensis type strain, CD36, should be carried out to find out the relevance of these polymorphisms.

While our data strongly imply that the observed reduced susceptibility to azole drugs was due to upregulation of CdCDR1 and CdCDR2 expression, the possibility still remains that additional resistance mechanisms could be involved. This possibility is currently being investigated using microarray analysis. In C. albicans, a transcriptional activator of the ABC transporter genes CDR1 and CDR2, named TAC1 (for transcriptional activator of CDR genes), has recently been identified (4). TAC1 alleles from azole-resistant strains, reintroduced in a TAC1 homologous mutant, were able to confer constitutive CDR1 and CDR2 upregulation, thus showing that azole resistance in the clinical strains had evolved from mutations in TAC1. Recently, the complete sequence of the C. dubliniensis genome has become available and a homologue of the TAC1 gene has been identified. It is likely that this homologue could be involved in the upregulation of CdCDR1 and CdCDR2 observed in the clinical isolates analyzed in the present study.

In conclusion, the analysis of susceptibility of multiple C. dubliniensis colonies from a single clinical sample revealed a significant degree of variation in susceptibility to azole drugs. The thorough analysis of matched clinical isolates belonging to C. dubliniensis genotype 3 showed that reduced susceptibility to azole drugs appeared to be associated only with increased energy-dependent efflux mechanisms mediated by the overexpression of the CdCDR1 and CdCDR2 genes. These results are in contrast to the mechanisms of azole resistance described to date for C. dubliniensis genotype 1, and they highlight the complexity and diversity of mechanisms by which C. dubliniensis isolates can develop resistance to azole drugs. Our previous studies were based on the analysis of the most common C. dubliniensis genotype 1. Since the majority of these isolates possess a defective CdCDR1 gene, we suggested that azole resistance mechanisms in C. dubliniensis were different from those in C. albicans. However, based on the data of the present study, it would appear that resistance mechanisms in C. dubliniensis genotypes 2, 3, and 4 may be more similar to those found in C. albicans.

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