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

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Running Title: Increased *CdCDR1* and *CdCDR2* expression in *C. dubliniensis*

Keywords: *C. dubliniensis*, genotype 3, azole resistance, *CdCDR1*, *CdCDR2*

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ABSTRACT

Candida dubliniensis is a recently identified yeast species primarily associated with oral carriage and infection in HIV-infected individuals. The species can be divided into at least four genotypes on the basis of the nucleotide sequence of the ITS 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 to azole 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 susceptibility 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 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 of azole 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 in azole resistance in *C. dubliniensis.*

Introduction

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 relapses in oral candidiasis in HIV-infected and AIDS patients following extensive therapeutic and prophylactic use of fluconazole (23, 36, 37)

A variety of molecular mechanisms by which *Candida* cells can develop resistance to azole drugs have been described previously. Azole resistance can be caused by increased expression of the target enzyme (cytochrome P450 lanosterol 14α-demethylase) resulting in increased cellular levels of the enzyme (18, 34) or as a result of point mutations which reduce its affinity for azole drugs (11, 13, 16, 17, 24, 29). Reduced accumulation of drug due to increased efflux is another mechanism commonly involved in azole 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 *C. 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 rRNA operon (8). Gee et al. found that isolates belonging to genotype 1 were predominant world-wide and mainly recovered from HIV-infected patients, while isolates belonging to genotypes 2, 3 and 4 were mainly recovered 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 in clinical azole-resistant genotype 1 *C. dubliniensis* isolates and in vitro-generated derivatives is primarily associated 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 *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 non-functional CdCdr1p protein.

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

Materials and methods

Candida 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 twelve single-colony isolates of *C. dubliniensis* present on the isolation plate were recovered simultaneously from this sample. On the basis of their susceptibility to azole 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 colour on CHROMagar Candida® medium and their identities confirmed using phenotypic and molecular techniques including biotyping using the API ID 32C yeast identification system (bioMérieux, Marcy l'Etoile, France) and PCR identification using the method developed by Donnelly et al (6). The *C. albicans* isolates were originally selected on the basis of their light green colour on CHROMagar Candida® medium and identified using the API ID 32C yeast identification system.

Candida dubliniensis and *C. albicans* clinical isolates were routinely cultured on potato dextrose agar (PDA; Oxoid, Basingstoke, UK) medium (pH 5.6) at 37˚C. For liquid culture, the isolates were grown in yeast-extract-peptone (YEPD) broth at 37˚C in an orbital shaker (Gallenkamp, Leicester, UK) at 200 rpm.

Susceptibility testing

Broth microdilution (BMD) susceptibility testing with azole drugs was carried out by the EUCAST broth microdilution method (5). All tests were carried out in duplicate.

DNA and RNA extractions and Southern and Northern blotting analyses

Total genomic DNA from the clinical isolates was prepared as described previously by Gallagher et al. (7). Fingerprinting and karyotype analyses were carried out as described by Gee et al. (8). Similarity coefficients (S_{AB}) based on band position obtained with the *C. dubliniensis*-specific fingerprinting probe Cd25 were calculated according to the formula $S_{AB}= 2E/ (2E+a+b)$, where E is the number of bands shared by isolate A and B, a is the number unique to A and b is the number of bands unique to B. An S_{AB} of 0.00 represents totally different patterns with no correlated bands, an S_{AB} of 1.00 represents identical patterns (32).

RNA extraction, electrophoresis, transfer and hybridisation were carried out as outlined by Moran et al. (21). Membranes were then exposed to BioMax MS film (Eastman Kodak Company, Rochester, New York, USA.) for 24 to 72 h. For normalization purposes, all membranes were hybridised with a probe homologous to the *C. dubliniensis* gene encoding translation elongation factor 3 (*CdTEF3*). Hybridisation levels were analyzed by scanning densitometry (GelWorks 1D Intermediate, Ultra-Violet Products Ltd., Cambridge, UK) and normalized with *CdTEF3* expression levels by dividing the intensity obtained for the gene of interest by the signal obtained for *CdTEF3.*

PCR amplification and cloning of *C. dubliniensis* DNA sequences

The amplification of *CdERG11* sequences was carried out using the primer pair ERG11F-ERG11R (26). The *CdMDR1* promoter sequences (positions –882 to +40 with respect to the

CdMDR1 start codon) were amplified by PCR with the primers PROF (5'-CAAAACGTGTTAGAATTGCGC-3') and PROR (5'-CTCTACCAACAAAACTATCTC-3'). The primer pair CdMDR1F-CdMDR1R (21) was used to amplify the *CdMDR1* ORF sequences. In order to reduce PCR errors, all PCR reactions were carried out using a proof-reading polymerase (Expand High Fidelity PCR System from Roche, Lewes, East Sussex, UK). The amplimers were used directly in sequencing reactions carried out by Lark Inc. (Saffron Walden, Essex, UK).

Biochemical analyses

Accumulation of [^{3}H]fluconazole in *C. dubliniensis* was assessed by the method of Sanglard et al (31) using cells grown to the mid-log phase in drug-free YNB. A time point of 20 minutes was used as this has been shown previously to represent steady-state conditions (21).

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

Glucose-induced efflux of rhodamine 6G (R6G) was determined in the absence of fluconazole using the method described by Pinjon et al. (23).

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 ITS region of the rRNA operon (data not shown). The isolates exhibited a range of susceptibility to fluconazole (MIC range 0.25 to 8 μg/ml), itraconazole (MIC range 0.06 to 0.5 μg/ml), ketoconazole (MIC range from 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) (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 twelve *C. dubliniensis* isolates, four representative isolates were selected for further study on the basis of their susceptibility to azole drugs. One isolate, CD519-8, was susceptible to all the azole drugs tested while the other three isolates (CD519-1, CD519-7 and CD519-14) showed reduced susceptibility to these drugs (Table 1). Although resistance to azole 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 susceptibility to 4NQO, flucytosine, crystal violet and 1,10 phenanthroline (data not shown). To determine the relatedness between these four isolates, they were analyzed by Southern hybridisation 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 from the *C. dubliniensis* type strain CD36 (S_{AB}= 0.24 to 0.26) which was used as a reference in fingerprinting procedures (Fig. 1.A). However, the Cd25-generated fingerprint

profile of CD519-14 (S_{AB}= 0.93) contained three polymorphic bands (indicated by arrows on Fig 1.A) which were not present in the fingerprint profiles of isolates CD519-1, CD519-7 and CD519-8 (S_{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 previously observed in *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 which was used as a reference (Fig. 1.B). 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 reduced 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 susceptibility to azole drugs observed in the clinical isolates CD519-1, CD519-7 and CD519-14 could be due to a defect in the enzyme sterol C5,6-desaturase, the non-saponifiable 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 mediating reduced susceptibility to azole drugs in the clinical

isolates CD519-1, CD519-7 and CD519-14, expression levels of the *CdERG11* gene in these isolates and the azole-susceptible clinical isolate CD519-8 were examined by Northern blot analysis. There was no significant elevation of *CdERG11* mRNA levels in any of the four clinical isolates examined (Fig. 2).

In addition, the *CdERG11* genes from all four isolates were amplified by PCR and sequenced directly. All four isolates had identical *CdERG11* nucleotide sequences which contained two homozygous polymorphisms (I188V and R499K) and one hetergozygous polymorphism (V402G) when compared with the recently published *CdERG11* sequence (25). To confirm this, the *CdERG11* genes from two isolates with differing susceptibility to azole drugs (i.e. CD519-1 and CD519-8) were expressed in the azole hypersusceptible *S. cerevisiae* strain YKKB-13 as described by Sanglard et al. (29). The susceptibility to itraconazole and fluconazole of the *S. cerevisiae* transformants expressing *CdERG11* genes from clinical isolates CD519-8 and CD519-1 were determined by BMD. There was no difference in susceptibility to azole drugs in transformants expressing the *CdERG11* gene from the azole-susceptible isolate CD519-8 compared to the azole susceptibility of the transformants expressing the *CdERG11* gene from the isolate with reduced susceptibility to azoles, CD519-1 (data not shown).

In order to determine if reduced accumulation of drug contributed to the difference in MICs observed between the CD519 clinical isolates, the accumulation levels of [^{3}H]fluconazole were examined in all four isolates. The isolates and the type strain CD36 were examined at a single time-point following 20 minutes exposure to [^{3}H]fluconazole (Fig. 3). The control strain CD36 which harbors a defective *CdCDR1* gene (20) accumulated 518 ± 0.7 cpm/10^7 cells. The three isolates (CD519-1, CD519-7 and CD519-14) with reduced susceptibility to azoles showed lower accumulation levels of [^{3}H]fluconazole compared to the azole-susceptible isolate CD519-8. The azole-susceptible CD519-8 yielded an average of 471 ± 29 cpm/10^7 cells following a 20 min

exposure to [^{3}H]fluconazole while isolates CD519-1, CD519-7 and CD519-14 which had reduced susceptibility to azoles yielded an average of 253 ± 3 cpm/10^7 cells, 272 ± 30 cpm/10^7 cells and 308 ± 57 cpm/10^7 cells respectively. This showed that in these three isolates, reduced susceptibility to azoles 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 to azole drugs. The method used in the present study directly assessed the efflux of R6G by measuring extracellular concentrations of R6G following the addition of glucose to energy-starved cells in the absence of fluconazole. Efflux of R6G from *C. dubliniensis* type strain CD36, harboring a non–functional *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 which reflected similar levels of R6G uptake (data not shown). However, in the presence of glucose, efflux of R6G from the azole-susceptible isolate CD519-8 was lower than the R6G efflux 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 harbour a functional *CdCDR1* gene. In order to confirm this, PCR amplification followed by RFLP analysis was carried out as Moran et al. have shown that the presence of a stop codon at position 756 in the *CdCDR1* ORF removes a restriction site for the enzyme *Ssp*I (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. The isolates CD519-1, CD519-7 and CD519-14, with reduced susceptibility to azole drugs, showed increased mRNA levels of the two multidrug resistance genes *CdCDR1* and *CdCDR2* compared to the azole-susceptible isolate CD519-8. However, this was not the case for the multidrug resistance gene *CdMDR1*, the expression of which was not detectable in any of the four clinical isolates (Fig. 2). *CdCDR1* expression was increased by approximately 5-fold, 2-fold and 6-fold in isolates CD519-1, CD519-7 and CD519-14 respectively. Expression of *CdCDR2* was increased by > 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 the primer pairs CdMDR1F-CdMDR1R and PROF-PROR respectively and 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 compared with 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 the clinical isolates CD519-1, CD519-7, CD519-8 and CD519-14 were identical. However, there were thirteen 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 which was identical to the susceptibility pattern of the transformant expressing the *CdMDR1* gene from *C. dubliniensis* type strain CD36. Both transformants showed decreased susceptibility to fluconazole, benomyl, cycloheximide and 1,10 phenanthroline compared with 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 genotype 1 *C. dubliniensis* isolates exhibiting reduced susceptibility / 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. 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 susceptibility to the azole drugs fluconazole, itraconazole, ketoconazole, voriconazole and posaconazole (Table 1).

The decreased susceptibility to azole drugs in 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) which suggested 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 accumulation of fluconazole (Fig. 3). This 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). Contrary 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 clinical isolates *C. dubliniensis* 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 accumulation of drug 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 was 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(s).

Although, five amino acid-altering polymorphisms (D32G, T68S, A105, T307I and E415K) were identified in the sequence of the *CdMDR1* ORF obtained from the four CD519 isolates, heterologous expression in *S. cerevisiae* YKKB13 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 in the promoter sequences of the *CdMDR1* genes from the CD519 clinical isolates compared to the promoter sequence obtained from the *C. dubliniensis* type strain CD36 (EMBL accession no. AJ227752) were identified, 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 strain 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-transporters genes *CDR1* and *CDR2* named *TAC1* (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 analysed 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 only associated 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 in genotype 1 *C. dubliniensis* and 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, i.e. genotype 1. Since the majority of these isolates possess a defective *CdCDR1* gene, this led us to suggest that azole resistance mechanisms in *C. dubliniensis* were different than in *C. albicans*. However, based on

the data of the present study, it would appear that resistance mechanisms in the *C. dubliniensi*s genotypes 2, 3 and 4 may be more similar to those found in *C. albicans*.

Acknowledgements

This study was supported by the Irish Health Research Board (grants 04-97 and RP/2002/6), by the Dublin Dental School and Hospital and by the BBSRC (SLK and CJJ).

References

1. **Albertson, G. D., M. Niimi, R. D. Cannon, and H. F. Jenkinson.** 1996. Multiple efflux mechanisms are involved in *Candida albicans* fluconazole resistance. Antimicrob. Agents Chemother. **40:**2835-2841.
2. **Ammerer, G.** 1983. Expression of genes in yeast using the *ADC1* promoter. Methods Enzymol. **101:**192-201.
3. **Coleman, D. C., D. J. Sullivan, D. E. Bennett, G. P. Moran, H. J. Barry, and D. B. Shanley.** 1997. Candidiasis: the emergence of a novel species, *Candida dubliniensis*. AIDS **11:**557-67.
4. **Coste, A., M. Karababa, F. Ischer, J. Bille, and D. Sanglard.** 2004. Presented at the 2004 Yeast Genetics and Molecular Biology Meeting, Seattle, Washington, USA.
5. **Cuenca-Estrella, M., W. Lee-Yang, M. A. Ciblak, B. A. Arthington-Skaggs, E. Mellado, D. W. Warnock, and J. L. Rodriguez-Tudela.** 2002. Comparative evaluation of NCCLS M27-A and EUCAST broth microdilution procedures for antifungal susceptibility testing of *Candida* species. Antimicrob Agents Chemother **46:**3644-7.
6. **Donnelly, S. M., D. J. Sullivan, D. B. Shanley, and D. C. Coleman.** 1999. Phylogenetic analysis and rapid identification of *Candida dubliniensis* based on analysis of *ACT1* intron and exon sequences. Microbiology **145 (Pt 8):**1871-82.
7. **Gallagher, P. J., D. E. Bennett, M. C. Henman, R. J. Russell, S. R. Flint, D. B. Shanley, and D. C. Coleman.** 1992. Reduced azole susceptibility of *Candida albicans* from HIV-positive patients and a derivative exhibiting colony morphology variation. J. Gen. Microbiol. **138:**1901-1911.

8. **Gee, S. F., S. Joly, D. R. Soll, J. F. Meis, P. E. Verweij, I. Polacheck, D. J. Sullivan, and D. C. Coleman.** 2002. Identification of four distinct genotypes of *Candida dubliniensis* and detection of microevolution in vitro and in vivo. J. Clin. Microbiol. **40:**556-74.

9. **Joly, S., C. Pujol, M. Rysz, K. Vargas, and D. R. Soll.** 1999. Development and characterization of complex DNA fingerprinting probes for the infectious yeast *Candida dubliniensis*. J. Clin. Microbiol. **37:**1035-44.

10. **Kelly, S. L., D. C. Lamb, A. J. Corran, B. C. Baldwin, and D. E. Kelly.** 1995. Mode of action and resistance to azole antifungals associated with the formation of 14 alpha-methylergosta-8,24(28)-dien-3 beta,6 alpha-diol. Biochem. Biophys. Res. Commun. **207:**910-5.

11. **Kelly, S. L., D. C. Lamb, and D. E. Kelly.** 1999. Y132H substitution in *Candida albicans* sterol 14alpha-demethylase confers fluconazole resistance by preventing binding to haem. FEMS Microbiol. Lett. **180:**171-5.

12. **Kelly, S. L., D. C. Lamb, D. E. Kelly, J. Loeffler, and H. Einsele.** 1996. Resistance to fluconazole and amphotericin in *Candida albicans* from AIDS patients. Lancet **348:**1523-1524.

13. **Kelly, S. L., D. C. Lamb, J. Loeffler, H. Einsele, and D. E. Kelly.** 1999. The G464S amino acid substitution in *Candida albicans* sterol 14alpha-demethylase causes fluconazole resistance in the clinic through reduced affinity. Biochem. Biophys. Res. Commun. **262:**174-9.

14. **Kirkpatrick, W. R., S. G. Revankar, R. K. McAtee, J. L. Lopez-Ribot, A. W. Fothergill, D. I. McCarthy, S. E. Sanche, R. A. Cantu, M. G. Rinaldi, and T. F. Patterson.** 1998. Detection of *Candida dubliniensis* in oropharyngeal samples from

human immunodeficiency virus-infected patients in North America by primary CHROMagar candida screening and susceptibility testing of isolates. J. Clin. Microbiol. **36:**3007-12.

15. **Lamb, D. C., D. E. Kelly, N. J. Manning, and S. L. Kelly.** 1997. Reduced intracellular accumulation of azole antifungal results in resistance in *Candida albicans* isolate NCPF 3363. FEMS Microbiol Lett. **147:**189-93.

16. **Lamb, D. C., D. E. Kelly, T. C. White, and S. L. Kelly.** 2000. The R467K amino acid substitution in *Candida albicans* sterol 14alpha-demethylase causes drug resistance through reduced affinity. Antimicrob. Agents Chemother. **44:**6 3-67.

17. **Marichal, P., L. Koymans, S. Willemsens, D. Bellens, P. Verhasselt, W. Luyten, M. Borgers, F. C. Ramaekers, F. C. Odds, and H. V. d. Bossche.** 1999. Contribution of mutations in the cytochrome P450 14alpha-demethylase (Erg11p, Cyp51p) to azole resistance in *Candida albicans*. Microbiology **145:**2701-2713.

18. **Marichal, P., H. Vanden Bossche, F. C. Odds, G. Nobels, D. W. Warnock, V. Timmerman, C. Van Broeckhoven, S. Fay, and P. Mose-Larsen.** 1997. Molecular biological characterization of an azole-resistant *Candida glabrata* isolate. Antimicrob. Agents Chemother. **41:**2229-37.

19. **Miyazaki, Y., A. Geber, H. Miyazaki, D. Falconer, T. Parkinson, C. Hitchcock, B. Grimberg, K. Nyswaner, and J. E. Bennett.** 1999. Cloning, sequencing, expression and allelic sequence diversity of *ERG3* (C-5 sterol desaturase gene) in *Candida albicans*. Gene **236:**43-51.

20. **Moran, G., D. Sullivan, J. Morschhauser, and D. Coleman.** 2002. The *Candida dubliniensis CdCDR1* Gene Is Not Essential for Fluconazole Resistance. Antimicrob. Agents Chemother. **46:**2829-41.

21. **Moran, G. P., D. Sanglard, S. M. Donnelly, D. B. Shanley, D. J. Sullivan, and D. C. Coleman.** 1998. Identification and expression of multidrug transporters responsible for fluconazole resistance in *Candida dubliniensis*. Antimicrob. Agents Chemother. **42:**1819-30.

22. **Moran, G. P., D. J. Sullivan, M. C. Henman, C. E. McCreary, B. J. Harrington, D. B. Shanley, and D. C. Coleman.** 1997. Antifungal drug susceptibilities of oral *Candida dubliniensis* isolates from human immunodeficiency virus (HIV)-infected and non-HIV-infected subjects and generation of stable fluconazole-resistant derivatives in vitro. Antimicrob. Agents Chemother. **41:**617-23.

23. **Muller, F.-M. C., M. Weig, J. Peter, and T. J. Walsh.** 2000. Azole cross-resistance to ketoconazole, fluconazole, itraconazole and voriconazole in clinical *Candida albicans* isolates from HIV-infected children with oropharyngeal candidosis. J. Antimicrob. Chemother. **46:**323-342.

24. **Perea, S., J. L. Lopez-Ribot, W. R. Kirkpatrick, R. K. McAtee, R. A. Santillan, M. Martinez, D. Calabrese, D. Sanglard, and T. F. Patterson.** 2001. Prevalence of molecular mechanisms of resistance to azole antifungal agents in *Candida albicans* strains displaying high-level fluconazole resistance isolated from human immunodeficiency virus-infected patients. Antimicrob. Agents Chemother. **45:**2676-84.

25. **Perea, S., J. L. Lopez-Ribot, B. L. Wickes, W. R. Kirkpatrick, O. P. Dib, S. P. Bachmann, S. M. Keller, M. Martinez, and T. F. Patterson.** 2002. Molecular Mechanisms of Fluconazole Resistance in *Candida dubliniensis* Isolates from Human Immunodeficiency Virus-Infected Patients with Oropharyngeal Candidiasis. Antimicrob. Agents Chemother. **46:**1695-703.

26. **Pinjon, E., G. P. Moran, C. J. Jackson, S. L. Kelly, D. Sanglard, D. C. Coleman, and D. J. Sullivan.** 2003. Molecular mechanisms of itraconazole resistance in *Candida dubliniensis*. Antimicrob Agents Chemother **47:**2424-37.

27. **Ponton, J., R. Ruchel, K. V. Clemons, D. C. Coleman, R. Grillot, J. Guarro, D. Aldebert, P. Ambroise-Thomas, J. Cano, A. J. Carrillo-Munoz, J. Gene, C. Pinel, D. A. Stevens, and D. J. Sullivan.** 2000. Emerging pathogens. Med Mycol **38 Suppl 1:**225-36.

28. **Ruhnke, M., A. Schmidt-Westhausen, and J. Morschhauser.** 2000. Development of simultaneous resistance to fluconazole in *Candida albicans* and *Candida dubliniensis* in a patient with AIDS. J Antimicrob Chemother **46:**291-5.

29. **Sanglard, D., F. Ischer, L. Koymans, and J. Bille.** 1998. Amino acid substitutions in the cytochrome P-450 lanosterol 14alpha-demethylase (*CYP51A1*) from azole-resistant *Candida albicans* clinical isolates contribute to resistance to azole antifungal agents. Antimicrob. Agents Chemother. **42:**241-53.

30. **Sanglard, D., F. Ischer, M. Monod, and J. Bille.** 1997. Cloning of *Candida albicans* genes conferring resistance to azole antifungal agents: characterization of *CDR2*, a new multidrug ABC transporter gene. Microbiology **143 (Pt 2):**405-16.

31. **Sanglard, D., K. Kuchler, F. Ischer, J. L. Pagani, M. Monod, and J. Bille.** 1995. Mechanisms of resistance to azole antifungal agents in *Candida albicans* isolates from AIDS patients involve specific multidrug transporters. Antimicrob. Agents Chemother. **39:**2378-86.

32. **Soll, D. R.** 2000. The ins and outs of DNA fingerprinting the infectious fungi Clin. Microbiol. Rev. **13:**332-370.

33. **Sullivan, D., and D. Coleman.** 1998. *Candida dubliniensis*: characteristics and identification. J. Clin. Microbiol. **36:**329-34.

34. **Vanden Bossche, H., P. Marichal, F. C. Odds, L. Le Jeune, and M. C. Coene.** 1992. Characterization of an azole-resistant *Candida glabrata* isolate. Antimicrob Agents Chemother **36:**2602-10.

35. **Venkateswarlu, K., D. W. Denning, N. J. Manning, and S. L. Kelly.** 1995. Resistance to fluconazole in *Candida albicans* from AIDS patients correlated with reduced intracellular accumulation of drug. FEMS Microbiol Lett **131:**337-41.

36. **White, T. C.** 1997. Increased mRNA levels of *ERG16, CDR*, and *MDR1* correlate with increases in azole resistance in *Candida albicans* isolates from a patient infected with human immunodeficiency virus. Antimicrob. Agents Chemother. **41:**1482-1487.

37. **White, T. C., M. A. Pfaller, M. G. Rinaldi, J. Smith, and S. W. Redding.** 1997. Stable azole drug resistance associated with a substrain of *Candida albicans* from an HIV-infected patient. Oral Dis. **3 Suppl 1:**S102-9.

38. **Wirsching, S., S. Michel, G. Köhler, and J. Morschhäuser.** 2000. Activation of the Multiple Drug Resistance gene *MDR1* in fluconazole-resistant, clinical *Candida albicans* strains is caused by mutations in a *trans*-regulatory factor. J. Bacteriol. **182:**400-404.

39. **Wirsching, S., G. P. Moran, D. J. Sullivan, D. C. Coleman, and J. Morschhauser.** 2001. MDR1-mediated drug resistance in *Candida dubliniensis*. Antimicrob. Agents Chemother. **45:**3416-21.

Figure 1:

DNA fingerprint patterns of *C. dubliniensis* clinical isolates CD519-1, CD519-7, CD519-8, CD519-14 and *C. dubliniensis* type strain CD36. (A) Cd25 hybridisation patterns of *Eco*RI-digested genomic DNA. The relative positions of molecular size reference markers (in kilobases) are indicated on the left of the panel. The positions of three polymorphic bands in the fingerprinting profile of CD519-14 are indicated by arrows (B) Electrophoretic karyotype patterns of *C. dubliniensis* clinical isolates CD519-1, CD519-7, CD519-8, CD519-14 and *C. dubliniensis* type strain CD36. The relative positions of molecular size reference markers (in megabases) are indicated on the left of the panel.

Figure 2:

Expression of *CdCDR1*, *CdCDR2*, *CdMDR1* and *CdERG11* analyzed by Northern blot of total RNA from isolates CD519-1, CD519-7, CD519-8, CD519-14, *C. dubliniensis* type strain CD36 and Cm2, a fluconazole-resistant genotype 1 *C. dubliniensis* isolate characterised by Moran et al. (18). Total RNA was extracted from cells growing exponentially in YEPD in the absence of azole drugs , and 15 μg were electrophoresed on a denaturing agarose gel. Following transfer to a nylon membrane, the blots were sequentially probed with radiolabeled DNA probes homologous to *CdMDR1, CdCDR1, CdCDR2, CdERG11* and the constitutively expressed *CdTEF3* gene. Expression of *CdTEF3* was used as an internal control for RNA loading.

Figure 3:

Accumulation of [^{3}H]fluconazole in *C. dubliniensis* clinical isolates with reduced susceptibility to azole drugs (CD519-1, CD519-7 and CD519-14) and an azole-susceptible clonally related isolate

(CD519-8). Accumulation levels were determined following 20 min incubation in the presence of [^{3}H]fluconazole. The *C. dubliniensis* type strain CD36 was used as a control in this experiment.

Figure 4:

Glucose-induced rhodamine 6G efflux from clinical isolates with reduced susceptibility to azole drugs (CD519-1, CD519-7 and CD519-14) and an azole-susceptible clonally related isolate (CD519-8). The *C. dubliniensis* type strain CD36 was used as a control in this experiment. Each bar indicates the standard error of the mean of three sets of experiments.

Figure 5:

Susceptibility of *S. cerevisiae* YKKB-13 (Δ*pdr5*) transformants harbouring cloned *CdMDR1* genes to fluconazole and metabolic inhibitors. *CdMDR1* alleles from *C. dubliniensis* isolates were amplified by PCR, cloned into the expression vector pAAH5 (2) and transformed into the *S. cerevisiae* strain YKKB-13. The transformants harbour the pAAH5 plasmid (YP5) and the *CdMDR1* genes from isolates CD36 (YGM3) and CD519-1 (EPY84). Each transformant was grown to exponential growth phase to a density of $2x10^7$ cells/ml and 4 µl were spotted in a dilution series on minimal agar medium plates containing fixed concentrations of fluconazole and metabolic inhibitors as indicated. Plates were incubated for 48 h at 30˚C

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

Isolate [c]	MIC (μg/ml) [a, b]				
	Fluconazole	Itraconazole	Ketoconazole	Voriconazole	Posaconazole
***C. dubliniensis*:**					
CD519-1	**8**	**0.5**	**0.125**	**0.125**	**0.25**
CD519-2	4	ND	ND	ND	ND
CD519-3	4	ND	ND	ND	ND
CD519-4	0.25	ND	ND	ND	ND
CD519-5	4	ND	ND	ND	ND
CD519-6	8	0.125	ND	ND	ND
CD519-7	**8**	**0.25**	**0.125**	**0.125**	**0.125**
CD519-8	**0.5**	**0.06**	**< 0.07**	**0.07**	**< 0.01**
CD519-10	4	ND	ND	ND	ND
CD519-11	2	0.125	ND	ND	ND
CD519-13	4	ND	ND	ND	ND
CD519-14	**8**	**0.5**	**0.125**	**0.125**	**0.125**
***C. albicans*:**					
CA1197-1	0.25	0.03	ND	ND	ND
CA1197-2	0.25	0.03	ND	ND	ND
CA1197-3	0.25	0.03	ND	ND	ND
CA1197-4	0.5	0.03	ND	ND	ND

[a] MICs were determined by broth microdilution as described in Materials and Methods
[b] ND: not determined
[c] Isolates in bold-type were selected for a more detailed phenotypic and molecular analysis

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

Isolate	Accumulated sterols [a]	Isolate	Accumulated sterols [a]
CD519-1	15.3 % ergosta-tetraenol	CD519-8	16.8 % ergosta-tetraenol
	61.2 % ergosterol		55.8 % ergosterol
	8.3 % episterol		6.7 % fecosterol
	2.8 % obtusifoliol		6.0 % episterol
	12.4 % unknown sterols		1.8 % obtusifoliol
			12.8 % unknown sterols
CD519-7	17.8 % ergosta-tetraenol	CD519-14	21.3 % ergosta-tetraenol
	61.5 % ergosterol		63.2 % ergosterol
	3.7 % fecosterol		2.9 % fecosterol
	7.5 % episterol		5.0 % episterol
	3.0 % obtusifoliol		3.1 % obtusifoliol
	6.7 % unknown sterols		4.5% unknown sterols

[a] Sterols were extracted from cells grown overnight in YEPD broth at 30˚C and analysed by gas chromatography and mass spectrometry as described in Materials and Methods

FIGURE 1

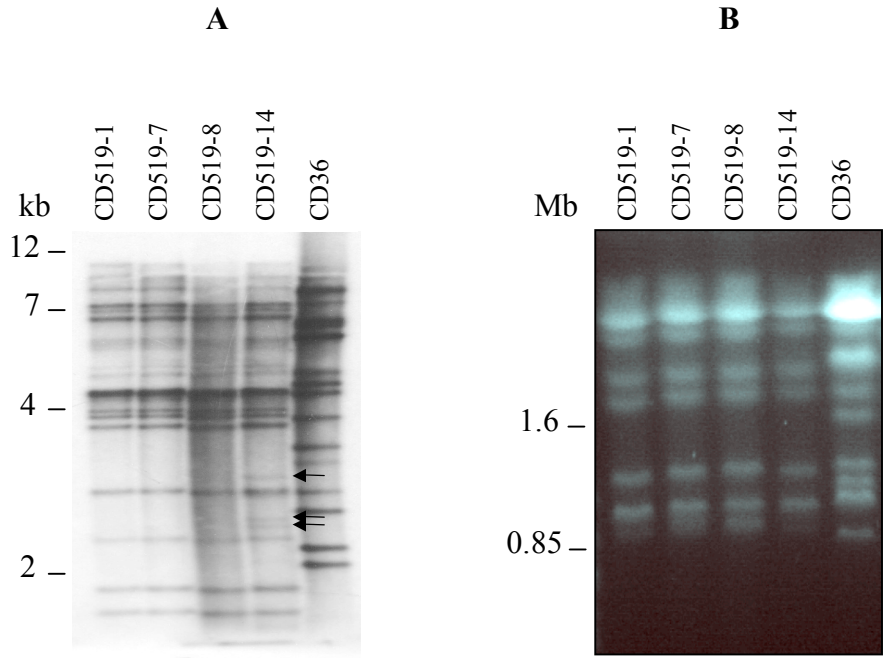


FIGURE 2

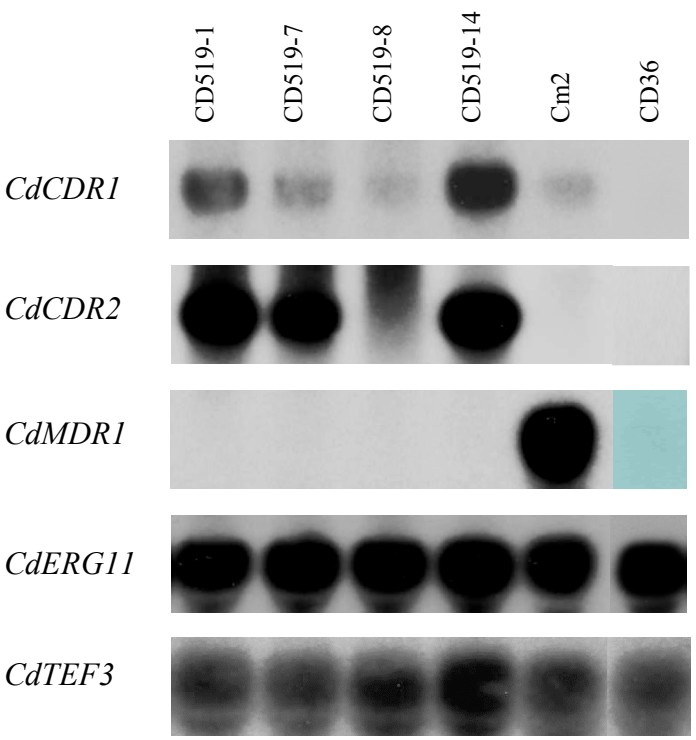

CD519-1
CD519-7
CD519-8
CD519-14
Cm2
CD36
CdCDR1
CdCDR2
CdMDR1
CdERG11
CdTEF3

FIGURE 3

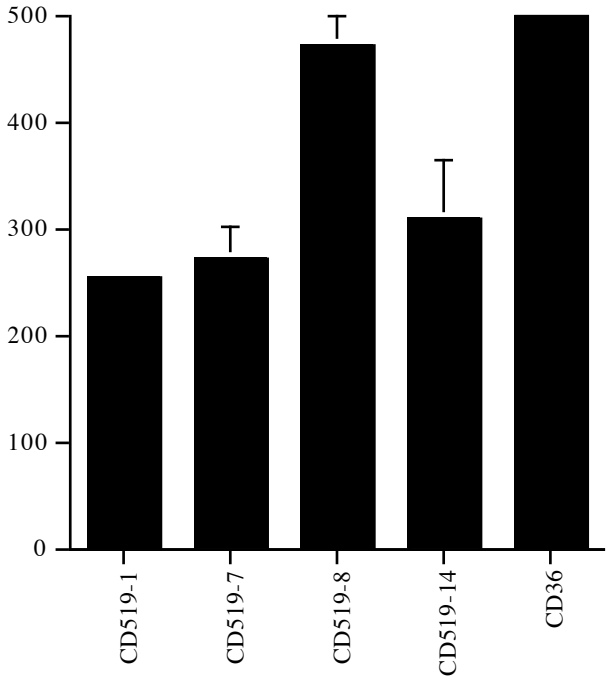


FIGURE 4

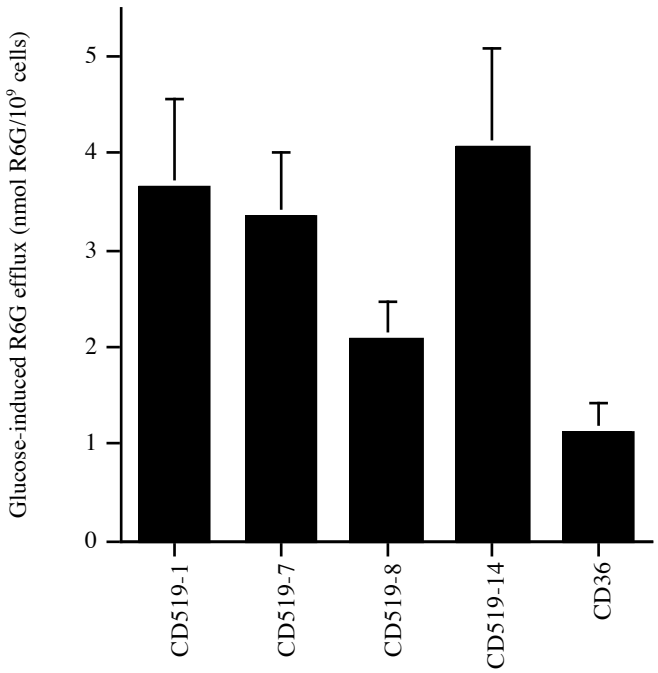
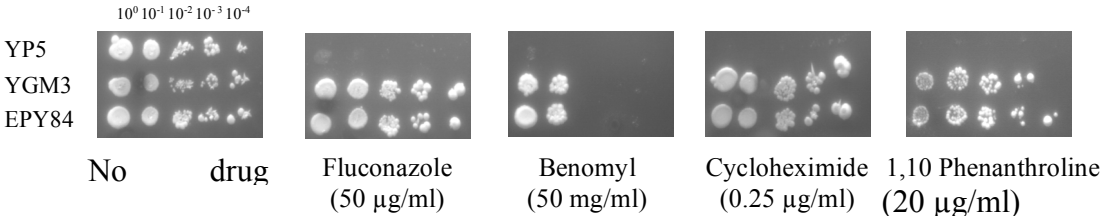

Glucose-induced R6G efflux (nmol R6G/10^9 cells)
0
1
2
3
4
5
CD519-1
CD519-7
CD519-8
CD519-14
CD36

FIGURE 5


10^0 10^{-1} 10^{-2} 10^{-3} 10^{-4}
YP5
YGM3
EPY84
No drug
Fluconazole (50 µg/ml)
Benomyl (50 mg/ml)
Cycloheximide (0.25 µg/ml)
1,10 Phenanthroline (20 µg/ml)