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Gain-of-Function Mutations in the Transcription Factor *MRR1* Are Responsible for Overexpression of the *MDR1* Efflux Pump in Fluconazole-Resistant *Candida dubliniensis* Strains▽†

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Candida dubliniensis*, a yeast that is closely related to *Candida albicans*, can rapidly develop resistance to the commonly used antifungal agent fluconazole in vitro and in vivo during antimycotic therapy. Fluconazole resistance in *C. dubliniensis* is usually caused by constitutive overexpression of the *MDR1* gene, which encodes a multidrug efflux pump of the major facilitator superfamily. The zinc cluster transcription factor Mrr1p has recently been shown to control *MDR1* expression in *C. albicans* in response to inducing stimuli, and gain-of-function mutations in the *MRR1* gene result in constitutive upregulation of the *MDR1* efflux pump. We identified a gene with a high degree of similarity to *C. albicans MRR1* (Ca*MRR1*) in the *C. dubliniensis* genome sequence. When *C. dubliniensis MRR1* (Cd*MRR1*) was expressed in *C. albicans mrr1*Δ mutants, it restored benomyl-inducible *MDR1* expression, demonstrating that Cd*MRR1* is the ortholog of Ca*MRR1*. To investigate whether *MDR1* overexpression in *C. dubliniensis* is caused by mutations in *MRR1*, we sequenced the *MRR1* alleles from a fluconazole-resistant, clinical *C. dubliniensis* isolate and a matched, fluconazole-susceptible isolate from the same patient as well as those from four in vitro-generated, fluconazole-resistant *C. dubliniensis* strains derived from two different *C. dubliniensis* isolates. We found that all five resistant strains contained single nucleotide substitutions or small in-frame deletions that resulted in amino acid changes in Mrr1p. Expression of these mutated alleles in *C. albicans* resulted in the constitutive activation of the *MDR1* promoter and multidrug resistance. Therefore, mutations in *MRR1* are the major cause of *MDR1* upregulation in both *C. albicans* and *C. dubliniensis*, demonstrating that the transcription factor Mrr1p plays a central role in the development of drug resistance in these human fungal pathogens.

The yeast *Candida dubliniensis* is closely related to *Candida albicans*, the major fungal pathogen of humans, but differs from it in certain phenotypic characteristics (34, 35). *C. dubliniensis* is less frequently associated with human disease and displays reduced virulence in animal models of infection. Comparative analyses of *C. albicans* and *C. dubliniensis* have provided insights into the molecular basis of differences in virulence and specific phenotypes between the two species. Genomic comparisons with DNA microarrays revealed that certain genes that contribute to the virulence of *C. albicans* are absent from the *C. dubliniensis* genome (15). Other genes are present in both species, but their expression is regulated differently. For example, differential regulation of the *NRG1* repressor is responsible for the higher propensity of *C. albicans* to switch from yeast to hyphal growth in response to environmental signals and for the production of chlamydospores in *C. dubliniensis*, but not in *C. albicans*, under certain conditions (17, 33). The recent sequencing of the genomes of both *C. albicans* (http://www.candidagenome.org) and *C. dubliniensis* (http://www.sanger.ac.uk/sequencing/Candida/dubliniensis) highly facil-itates the functional analysis and comparison of the role of specific genes in the two species.

Candida infections are commonly treated with the antimycotic agent fluconazole, which inhibits the biosynthesis of ergosterol, the major sterol in the fungal cell membrane. Like *C. albicans*, *C. dubliniensis* can become resistant to fluconazole during antifungal therapy, and stable fluconazole resistance can also be readily induced in vitro following exposure to the drug (11, 13, 16, 18, 19, 22, 24, 30). Constitutive overexpression of two types of multidrug efflux pumps, the ATP-binding cassette (ABC) transporters Cdr1p and Cdr2p or the major facilitator Mdr1p, is a major cause of resistance to fluconazole and other, structurally unrelated toxic compounds in *C. albicans* (20, 23, 31). *C. dubliniensis* contains homologs of these genes (18). However, the *CDR1* gene is inactivated by a point mutation in many *C. dubliniensis* strains and *CDR2* is rarely expressed; therefore, *MDR1* overexpression is the major mechanism of fluconazole resistance in this species (16, 18, 22, 24, 36, 38).

It has been known for quite a while that *MDR1* overexpression in fluconazole-resistant *C. albicans* isolates is caused by mutations in a *trans*-regulatory factor (38), but the molecular basis for *MDR1* upregulation in such strains has only recently been elucidated (21). The zinc cluster transcription factor Mrr1p mediates the expression of *MDR1* in response to inducing chemicals, and all *MDR1*-overexpressing clinical and in

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vitro-generated, fluconazole-resistant *C. albicans* strains that have been investigated so far contain gain-of-function mutations in the *MRR1* gene that result in the constitutive activity of this transcription factor (5, 21). As *MDR1* is overexpressed in almost all *C. dubliniensis* strains with reduced fluconazole susceptibility, we investigated whether *C. dubliniensis* contains an ortholog of the transcription factor *MRR1* and if *MDR1* overexpression in *C. dubliniensis* is also caused by mutations in this transcription factor.

MATERIALS AND METHODS

Strains and growth conditions. The strains used in this study are listed in Table 1. All strains were stored as frozen stocks with 15% glycerol at −80°C and were subcultured on YPD agar plates (10 g yeast extract, 20 g peptone, 20 g glucose, 15 g agar per liter) at 30°C. For routine growth of the strains, YPD liquid medium was used. For induction of the *MDR1* promoter with benomyl, overnight cultures of reporter strains were diluted 10^{-2} in two flasks with fresh YPD medium and were grown for 3 h. Benomyl (50 μg ml^{-1}) was then added to one of the cultures and the cells were grown for an additional hour. The fluorescence of the cells was quantified by fluorescence-activated cell sorter (FACS) analysis.

Plasmid constructions. Plasmid pZCF36E1, which contains the *MRR1* gene from *C. albicans* strain SC5314 under the control of the *ADH1* promoter in the vector pBC, has been described previously (21). An ApaI-SacII fragment containing the complete insert from this plasmid was cloned into the vector pBluescript to obtain pZCF36E2. Substitution of the *MRR1*^{F5} allele from the fluconazole-resistant isolate F5, which contains a P683S gain-of-function mutation but is otherwise identical to *MRR1* from strain SC5314, for the *MRR1* open reading frame in pZCF36E2 resulted in pZCF36E3. The coding regions of the *MRR1* alleles of *C. dubliniensis* strains CM1, CM2, CD57, CD57A, CD57B, CD51-IIA, and CD51-IIB were amplified with primers CdMRR1-1 (5′-GTTATTCGTATTCTCGAGAAATGTCAGTTGCC-3′) and CdMRR1-2 (5′-CAAATCACCAGATCTATTTCAATTGGTAAAAAG-3′), digested at the introduced XhoI and BglII sites (underlined), and cloned between the same sites of plasmid pADH1E1 (25) to result in plasmids pCdMRR1E1, pCdMRR1E2, pCd MRR1E3, pCdMRR1E4, pCdMRR1E5, pCdMRR1E6, and pCdMRR1E7, respectively (Fig. 1A). The *MRR1* allele from *C. dubliniensis* strain CD51-II was cloned in the same way, and sequencing showed that it was identical to the *MRR1* allele of strain CM1 contained in pCdMRR1E1.

***C. albicans* transformation.** *C. albicans* strains were transformed by electroporation (12) with the gel-purified ApaI-SacII fragments from the plasmids described above. Nourseothricin-resistant transformants were selected on YPD agar plates containing 200 μg ml^{-1} nourseothricin (Werner Bioagents, Jena, Germany), as described previously (26). Single-copy integration of all constructs was confirmed by Southern hybridization.

Isolation of genomic DNA and Southern hybridization. Genomic DNA was isolated from the *C. albicans* and *C. dubliniensis* strains as described previously (14). To confirm the specific integration of the expression cassettes into the *C. albicans* genome, the DNA of the parental strains and transformants was digested with SpeI, separated on a 1% agarose gel, and after ethidium bromide staining, transferred by vacuum blotting onto a nylon membrane and fixed by UV cross-linking. Southern hybridization with enhanced chemiluminescence-labeled probes was performed with the Amersham ECL direct nucleic acid labeling and detection system (GE Healthcare, Braunschweig, Germany), according to the instructions of the manufacturer. The P_{ADH1} and 3′ *ADH1* fragments from pADH1E1 were used as probes. Correct integration of the cassettes into one of the genomic *ADH1* alleles resulted in the appearance of a new 8.5-kb SpeI fragment, in addition to the 3.3-kb wild-type fragment.

Drug susceptibility tests. Stock solutions of the drugs were prepared as follows. Fluconazole (5 mg ml^{-1}) was dissolved in water, while cerulenin (5 mg ml^{-1}) and brefeldin A (5 mg ml^{-1}) were dissolved in dimethyl sulfoxide. In the assays, serial twofold dilutions in the assay medium were prepared from the following initial concentrations: cerulenin, 50 μg ml^{-1}; brefeldin A, 200 μg ml^{-1}; and fluconazole, 200 μg ml^{-1}. Susceptibility tests were carried out in high-resolution medium (14.67 g HR medium [Oxoid GmbH, Wesel, Germany], 1 g $NaHCO_3$, 0.2 M phosphate buffer [pH 7.2]), using a previously described microdilution method (29).

FACS analysis. FACS analysis was performed with a FACSCalibur cytometry system equipped with an argon laser emitting at 488 nm (Becton Dickinson, Heidelberg, Germany). Fluorescence was measured on the FL1 fluorescence channel equipped with a 530-nm band-pass filter. Twenty thousand cells were analyzed per sample. Fluorescence data were collected by using logarithmic amplifiers. The mean fluorescence values were determined with CellQuest Pro software (Becton Dickinson).

RESULTS

Cd*MRR1* encodes the *C. dubliniensis* ortholog of the *C. albicans* multidrug resistance regulator Ca*MRR1*. A BLAST search of the *C. dubliniensis* genome sequence identified an open reading frame (Cd36_85850) whose deduced amino acid sequence exhibits 91% identity with Mrr1p of *C. albicans* (CaMrr1p), suggesting that this gene encodes the Mrr1p ortholog in *C. dubliniensis* (see Fig. S1 in the supplemental material). To test whether *C. dubliniensis* Mrr1p (CdMrr1p) could complement a *C. albicans mrr1*Δ mutant, we cloned the complete *C. dubliniensis MRR1* (Cd*MRR1*) coding sequence from the fluconazole-susceptible *C. dubliniensis* isolate CM1 and expressed it under the control of the *C. albicans ADH1* promoter in an *mrr1*Δ mutant that contained a P_{MDR1}-*GFP* (green fluorescent protein) reporter gene fusion (Fig. 1A). The *MDR1* promoter is inactive under standard growth conditions, but it can be induced by certain toxic chemicals, like benomyl, in an Mrr1p-dependent fashion (8–10, 21, 28, 32). As can be seen in Fig. 1B, Cd*MRR1* restored benomyl-induced *MDR1* expression in the *mrr1*Δ mutant with equal efficiency as *C. albicans MRR1* (Ca*MRR1*) expressed from the same promoter. These results demonstrated that, like Ca*MRR1*, Cd*MRR1* controls the expression of the *MDR1* efflux pump.

***MDR1*-overexpressing, fluconazole-resistant *C. dubliniensis* strains contain mutations in *MRR1*.** Gain-of-function mutations in *MRR1* are responsible for the constitutive *MDR1* upregulation in fluconazole-resistant *C. albicans* strains (5, 21). We therefore investigated whether *MDR1* overexpression in fluconazole-resistant *C. dubliniensis* strains is also caused by mutations in *MRR1*. For this purpose, we cloned and sequenced the *MRR1* alleles from an *MDR1*-overexpressing, fluconazole-resistant *C. dubliniensis* clinical isolate and four resistant strains that were generated in vitro from two different susceptible isolates by serial passage in the presence of increasing concentrations of fluconazole (18, 19).

The fluconazole-resistant isolate CM2 was obtained from the same patient as the susceptible isolate CM1, and karyotype analysis and DNA fingerprinting had demonstrated the genetic relatedness of these isolates (37, 38). The cloned *MRR1* allele of isolate CM1 was identical to that found in the *C. dubliniensis* genome sequence. Isolate CM2 contained the same allele, except for a G-A exchange at position 2597 that caused a C866Y mutation in CdMrr1p. Direct sequencing of the relevant part of the amplified PCR products confirmed the absence of the mutation in isolate CM1 and its presence in both *MRR1* alleles of isolate CM2.

The *MRR1* allele that was cloned from the fluconazole-susceptible isolate CD57 exhibited several differences from that in the *C. dubliniensis* genome sequence: three silent substitutions (G933A, G1419A, A2721G) as well as two nonsynonymous substitutions, G223A and T2503A, which resulted in the exchanges G75R and S835T, respectively, in the encoded protein. The same allele was cloned from the two in vitro-generated, fluconazole-resistant derivatives CD57A and CD57B. However, CD57A contained an additional C1784A

TABLE 1. Strains used in this study

Strain	Parental strain	Relevant characteristic or genotype	Reference or source
C. dubliniensis strains			
CM1		FLU^S isolate from patient 1	18, 19
CM2		*MDR1*-overexpressing FLU^R isolate from patient 1	18, 19
CD57		FLU^S isolate from patient 15	18, 19
CD57A	CD57	In vitro-generated, *MDR1*-overexpressing FLU^R strain	18, 19
CD57B	CD57	In vitro-generated, *MDR1*-overexpressing FLU^R strain	18, 19
CD51-II		FLU^S isolate from patient 8	18, 19
CD51-IIA	CD51-II	In vitro-generated, *MDR1*-overexpressing FLU^R strain	18, 19
CD51-IIB	CD51-II	In vitro-generated, *MDR1*-overexpressing FLU^R strain	18, 19
C. albicans strains			
SC5314		Prototrophic wild-type strain	7
SCMRR1M4A and -B	SC5314	*mrr1*Δ::*FRT*/*mrr1*Δ::*FRT*	21
SCMRR1M4E2A	SCMRR1M4A	*mrr1*Δ::*FRT*/*mrr1*Δ::*FRT* *ADH1*/*adh1*::P_{ADH1}-Ca*MRR1-caSAT1*	This study
SCMRR1M4E2B	SCMRR1M4B	*mrr1*Δ::*FRT*/*mrr1*Δ::*FRT* *ADH1*/*adh1*::P_{ADH1}-Ca*MRR1-caSAT1*	This study
SCMRR1M4E3A	SCMRR1M4A	*mrr1*Δ::*FRT*/*mrr1*Δ::*FRT* *ADH1*/*adh1*::P_{ADH1}-Ca*MRR1*^{F5}*-caSAT1*	This study
SCMRR1M4E3B	SCMRR1M4B	*mrr1*Δ::*FRT*/*mrr1*Δ::*FRT* *ADH1*/*adh1*::P_{ADH1}-Ca*MRR1*^{F5}*-caSAT1*	This study
SCMRR1M4CdE1A	SCMRR1M4A	*mrr1*Δ::*FRT*/*mrr1*Δ::*FRT* *ADH1*/*adh1*::P_{ADH1}-Cd*MRR1*^{CM1}*-caSAT1*	This study
SCMRR1M4CdE1B	SCMRR1M4B	*mrr1*Δ::*FRT*/*mrr1*Δ::*FRT* *ADH1*/*adh1*::P_{ADH1}-Cd*MRR1*^{CM1}*-caSAT1*	This study
SCMRR1M4CdE2A	SCMRR1M4A	*mrr1*Δ::*FRT*/*mrr1*Δ::*FRT* *ADH1*/*adh1*::P_{ADH1}-Cd*MRR1*^{CM2}*-caSAT1*	This study
SCMRR1M4CdE2B	SCMRR1M4B	*mrr1*Δ::*FRT*/*mrr1*Δ::*FRT* *ADH1*/*adh1*::P_{ADH1}-Cd*MRR1*^{CM2}*-caSAT1*	This study
SCMRR1M4CdE3A	SCMRR1M4A	*mrr1*Δ::*FRT*/*mrr1*Δ::*FRT* *ADH1*/*adh1*::P_{ADH1}-Cd*MRR1*^{CD57}*-caSAT1*	This study
SCMRR1M4CdE3B	SCMRR1M4B	*mrr1*Δ::*FRT*/*mrr1*Δ::*FRT* *ADH1*/*adh1*::P_{ADH1}-Cd*MRR1*^{CD57}*-caSAT1*	This study
SCMRR1M4CdE4A	SCMRR1M4A	*mrr1*Δ::*FRT*/*mrr1*Δ::*FRT* *ADH1*/*adh1*::P_{ADH1}-Cd*MRR1*^{CD57A}*-caSAT1*	This study
SCMRR1M4CdE4B	SCMRR1M4B	*mrr1*Δ::*FRT*/*mrr1*Δ::*FRT* *ADH1*/*adh1*::P_{ADH1}-Cd*MRR1*^{CD57A}*-caSAT1*	This study
SCMRR1M4CdE5A	SCMRR1M4A	*mrr1*Δ::*FRT*/*mrr1*Δ::*FRT* *ADH1*/*adh1*::P_{ADH1}-Cd*MRR1*^{CD57B}*-caSAT1*	This study
SCMRR1M4CdE5B	SCMRR1M4B	*mrr1*Δ::*FRT*/*mrr1*Δ::*FRT* *ADH1*/*adh1*::P_{ADH1}-Cd*MRR1*^{CD57B}*-caSAT1*	This study
SCMRR1M4CdE6A	SCMRR1M4A	*mrr1*Δ::*FRT*/*mrr1*Δ::*FRT* *ADH1*/*adh1*::P_{ADH1}-Cd*MRR1*$^{CD51\text{-}IIA}$*-caSAT1*	This study
SCMRR1M4CdE6B	SCMRR1M4B	*mrr1*Δ::*FRT*/*mrr1*Δ::*FRT* *ADH1*/*adh1*::P_{ADH1}-Cd*MRR1*$^{CD51\text{-}IIA}$*-caSAT1*	This study
SCMRR1M4CdE7A	SCMRR1M4A	*mrr1*Δ::*FRT*/*mrr1*Δ::*FRT* *ADH1*/*adh1*::P_{ADH1}-Cd*MRR1*$^{CD51\text{-}IIB}$*-caSAT1*	This study
SCMRR1M4CdE7B	SCMRR1M4B	*mrr1*Δ::*FRT*/*mrr1*Δ::*FRT* *ADH1*/*adh1*::P_{ADH1}-Cd*MRR1*$^{CD51\text{-}IIB}$*-caSAT1*	This study
CAG48MRR1M4B	SC5314	*mrr1*Δ::*FRT*/*mrr1*Δ::*FRT MDR1*/*mdr1*::P_{MDR1}-*GFP-URA3*	21
CAG48MRR1M4E2B1 and -2	CAG48MRR1M4B	*mrr1*Δ::*FRT*/*mrr1*Δ::*FRT MDR1*/*mdr1*::P_{MDR1}-*GFP-URA3* *ADH1*/*adh1*::P_{ADH1}-Ca*MRR1-caSAT1*	This study
CAG48MRR1M4E3B1 and -2	CAG48MRR1M4B	*mrr1*Δ::*FRT*/*mrr1*Δ::*FRT MDR1*/*mdr1*::P_{MDR1}-*GFP-URA3* *ADH1*/*adh1*::P_{ADH1}-Ca*MRR1*^{F5}*-caSAT1*	This study
CAG48MRR1M4CdE1B1 and -2	CAG48MRR1M4B	*mrr1*Δ::*FRT*/*mrr1*Δ::*FRT MDR1*/*mdr1*::P_{MDR1}-*GFP-URA3* *ADH1*/*adh1*::P_{ADH1}-Cd*MRR1*^{CM1}*-caSAT1*	This study
CAG48MRR1M4CdE2B1 and -2	CAG48MRR1M4B	*mrr1*Δ::*FRT*/*mrr1*Δ::*FRT MDR1*/*mdr1*::P_{MDR1}-*GFP-URA3* *ADH1*/*adh1*::P_{ADH1}-Cd*MRR1*^{CM2}*-caSAT1*	This study
CAG48MRR1M4CdE3B1 and -2	CAG48MRR1M4B	*mrr1*Δ::*FRT*/*mrr1*Δ::*FRT MDR1*/*mdr1*::P_{MDR1}-*GFP-URA3* *ADH1*/*adh1*::P_{ADH1}-Cd*MRR1*^{CD57}*-caSAT1*	This study
CAG48MRR1M4CdE4B1 and -2	CAG48MRR1M4B	*mrr1*Δ::*FRT*/*mrr1*Δ::*FRT MDR1*/*mdr1*::P_{MDR1}-*GFP-URA3* *ADH1*/*adh1*::P_{ADH1}-Cd*MRR1*^{CD57A}*-caSAT1*	This study
CAG48MRR1M4CdE5B1 and -2	CAG48MRR1M4B	*mrr1*Δ::*FRT*/*mrr1*Δ::*FRT MDR1*/*mdr1*::P_{MDR1}-*GFP-URA3* *ADH1*/*adh1*::P_{ADH1}-Cd*MRR1*^{CD57B}*-caSAT1*	This study
CAG48MRR1M4CdE6B1 and -2	CAG48MRR1M4B	*mrr1*Δ::*FRT*/*mrr1*Δ::*FRT MDR1*/*mdr1*::P_{MDR1}-*GFP-URA3* *ADH1*/*adh1*::P_{ADH1}-Cd*MRR1*$^{CD51\text{-}IIA}$*-caSAT1*	This study
CAG48MRR1M4CdE7B1 and -2	CAG48MRR1M4B	*mrr1*Δ::*FRT*/*mrr1*Δ::*FRT MDR1*/*mdr1*::P_{MDR1}-*GFP-URA3* *ADH1*/*adh1*::P_{ADH1}-Cd*MRR1*$^{CD51\text{-}IIB}$*-caSAT1*	This study

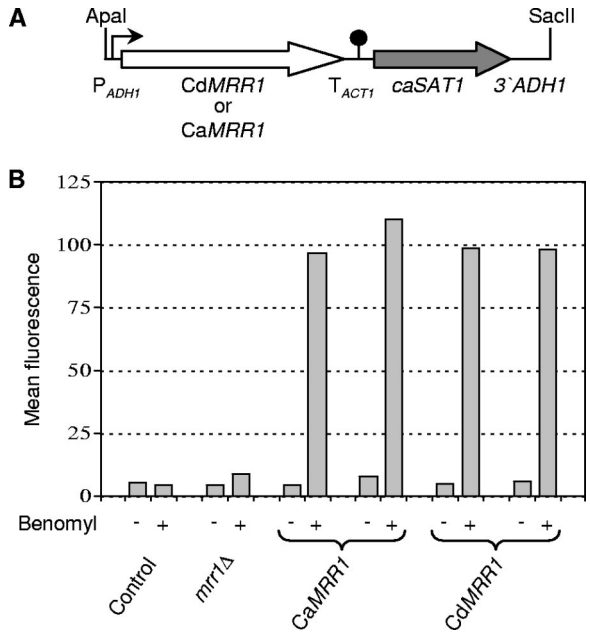

FIG. 1. Cd*MRR1* complements the defect in inducible *MDR1* expression of a *C. albicans mrr1*Δ mutant. (A) Structure of the cassette that was used to express Cd*MRR1* or Ca*MRR1* (white arrow) from the *ADH1* promoter (P_{ADH1}, bent arrow) after integration into the *C. albicans* genome with the help of the *caSAT1* marker (gray arrow). T_{ACT1}, transcription termination sequence of the *ACT1* gene (filled circle). (B) Fluorescence of *C. albicans* strains carrying a P_{MDR1}-*GFP* reporter fusion in an *mrr1*Δ background and expressing either Ca*MRR1* or Cd*MRR1* from the *ADH1* promoter. Two independent transformants of parental strain CAG48MRR1M4B (*mrr1*Δ) were used in each case. Strain SC5314 (control), which does not contain the *GFP* gene, was included to control for background fluorescence. The strains were grown in the presence (+) or the absence (−) of benomyl, and the mean fluorescence of the cells was determined by flow cytometry, as detailed in Materials and Methods.

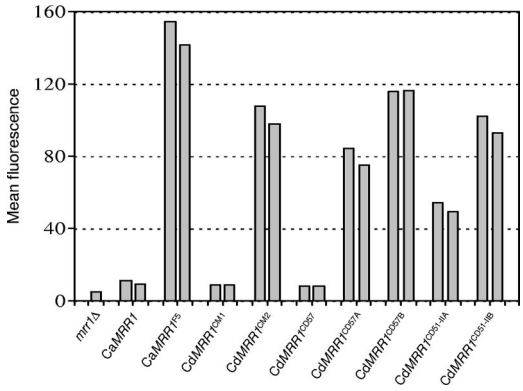

FIG. 2. The mutated Cd*MRR1* alleles constitutively activate the *MDR1* promoter. The indicated Ca*MRR1* and Cd*MRR1* alleles were expressed under the control of the *ADH1* promoter in a *C. albicans mrr1*Δ mutant carrying a P_{MDR1}-*GFP* reporter fusion (*mrr1*Δ). Strains were grown to log phase in YPD medium, and the mean fluorescence of the cells was determined by flow cytometry. Two independent transformants expressing the various *MRR1* alleles were used in each case.

substitution, which resulted in an S595Y mutation in CdMrr1p, and CD57B contained an additional C1121T exchange, which resulted in a T374I mutation.

The *MRR1* allele that was cloned from isolate CD51-II was identical to the one found in isolate CM1 and in the *C. dubliniensis* genome sequence. The same allele was also recovered from its two in vitro-generated, fluconazole-resistant derivatives, CD51-IIA and CD51-IIB, except that both contained small in-frame deletions in the *MRR1* coding sequence. In strain CD51-IIA, 36 nucleotides from positions 2959 to 2994, encoding amino acids D987 to I998, were deleted; and strain CD51-IIB lacked 3 nucleotides from positions 2952 to 2954, resulting in the deletion of T985 in CdMrr1p. Direct sequencing of the amplified PCR products demonstrated that all four in vitro-generated, resistant strains had become homozygous for the mutations and that the mutations were absent from the *MRR1* alleles of the parental strains. Therefore, *MDR1* overexpression correlated with mutations in *MRR1* in all five fluconazole-resistant *C. dubliniensis* strains investigated.

Gain-of-function mutations in Cd*MRR1* cause constitutive *MDR1* overexpression and multidrug resistance. The presence of *MRR1* mutations in *MDR1*-overexpressing, fluconazole-resistant *C. dubliniensis* strains suggested that these mutations resulted in constitutive activity of the transcription factor. To confirm this hypothesis, we expressed the different Cd*MRR1* alleles in the *C. albicans mrr1*Δ mutant carrying the P_{MDR1}-*GFP* reporter fusion. Except for the putative gain-of-function mutations, the *MRR1* alleles from strains CM2, CD51-IIA, and CD51-IIB were identical to the *MRR1* allele from isolate CM1, which was used for comparison. As the *MRR1* alleles from strains CD57A and CD57B contained additional polymorphisms (see above), the otherwise identical but nonmutated allele from the parental strain, strain CD57, was used as an additional control. For comparison, we also expressed the wild-type *MRR1* allele from *C. albicans* strain SC5314 and the constitutively active $MRR1^{F5}$ allele with the P683S mutation (21) in the same way from the *ADH1* promoter. As can be seen in Fig. 2, the expression of all five mutated Cd*MRR1* alleles (Cd$MRR1^{CM2}$, Cd$MRR1^{CD57A}$, Cd$MRR1^{CD57B}$, Cd$MRR1^{CD51-IIA}$, Cd$MRR1^{CD51-IIB}$) resulted in the constitutive activation of the *MDR1* promoter, whereas the corresponding nonmutated alleles Cd$MRR1^{CM1}$ and Cd$MRR1^{CD57}$ did not induce *MDR1* expression. Therefore, the mutations that had occurred in the Cd*MRR1* alleles of *MDR1*-overexpressing, fluconazole-resistant *C. dubliniensis* strains were indeed gain-of-function mutations that rendered the transcription factor constitutively active.

In a complementary approach to investigate the effect of the Cd*MRR1* mutations on drug resistance, the same alleles described above were also introduced into *mrr1*Δ mutants of the *C. albicans* wild-type strain SC5314 and the susceptibilities of the transformants to fluconazole and other metabolic inhibitors to which *MDR1* overexpression confers resistance were tested. Figure 3 shows that expression of the five mutated Cd*MRR1* alleles conferred 4- to 8-fold increased resistance to fluconazole, 4- to 16-fold increased resistance to cerulenin, and 2- to 8-fold increased resistance to brefeldin A, while expression of the corresponding nonmutated Cd$MRR1^{CM1}$ and Cd$MRR1^{CD57}$ alleles did not affect the susceptibilities of the parental strains to these compounds. These results demonstrated that the Cd*MRR1* mutations conferred multidrug resistance when they were expressed in *C. albicans*, indicating that they were responsible for drug resistance

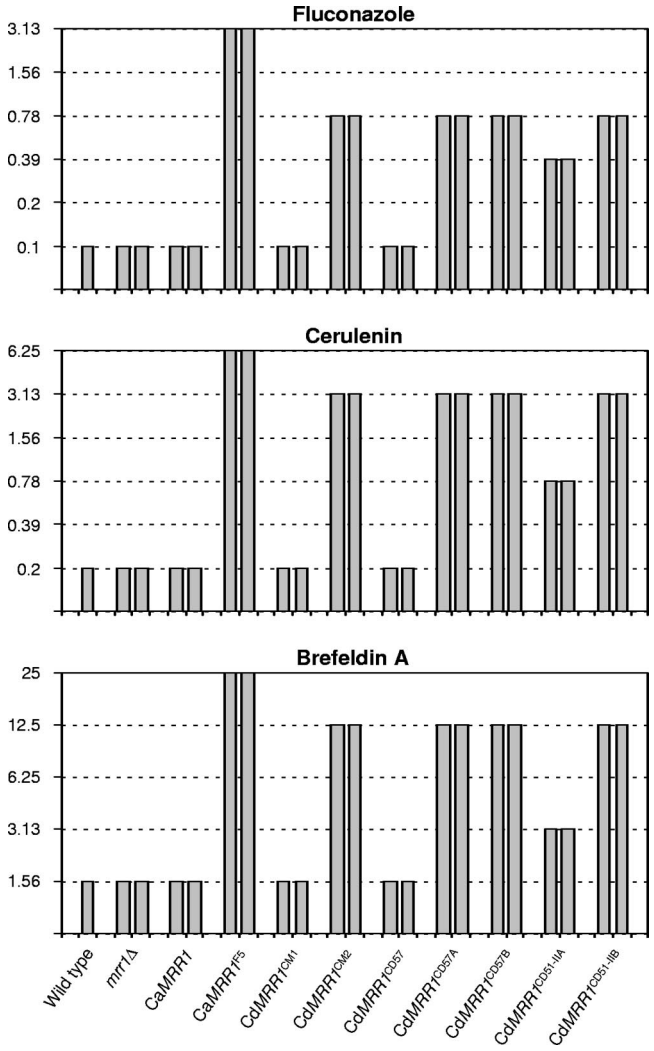

FIG. 3. MICs (in $\mu g\ ml^{-1}$) of fluconazole, cerulenin, and brefeldin A for wild-type parental strain SC5314, two independently constructed homozygous *mrr1*Δ mutants, and transformants expressing the indicated *MRR1* alleles under the control of the *ADH1* promoter.

in the *MDR1*-overexpressing *C. dubliniensis* strains in which these mutations had been selected by the presence of fluconazole.

DISCUSSION

The transcription factor Mrr1p has only recently been identified as the central regulator of the *MDR1* efflux pump in *C. albicans* (21). All *MDR1*-overexpressing, fluconazole-resistant *C. albicans* strains investigated to date (nine clinical isolates and five in vitro-generated strains) contain gain-of-function mutations in this transcription factor that cause the constitutive upregulation of *MDR1* (5, 21). In contrast to *C. albicans*, in which overexpression of the ABC transporters *CDR1* and *CDR2* is an even more frequent mechanism of drug resistance (22), *MDR1* overexpression is the major cause of fluconazole resistance in almost all resistant *C. dubliniensis* strains (16, 18, 19, 36, 38). An initial study suggested that *C. dubliniensis* develops fluconazole resistance more readily than *C. albicans* when it is exposed to increasing concentrations of the drug in vitro (19). Although fluconazole-resistant *C. albicans* strains, including *MDR1* overexpressing strains, were also later successfully generated in vitro when other strains were used as the starting material (1, 3, 4, 6, 27, 39), the former findings raised the possibility that *C. dubliniensis* may have additional mechanisms to rapidly achieve constitutive *MDR1* overexpression. However, in the present study, we found that all five *MDR1*-overexpressing *C. dubliniensis* strains that were available to us, one clinical isolate and four in vitro-generated strains described by Moran et al. (18, 19), contained mutations in Cd*MRR1*, the *C. dubliniensis* ortholog of Ca*MRR1*. Therefore, mutations in this transcription factor seem to be the major, if not the sole, cause of *MDR1* overexpression in both *C. albicans* and *C. dubliniensis*. Figure 4 shows the positions of the five mutations identified in the CdMrr1p protein, along with the positions at which gain-of-function mutations have previously been found in CaMrr1p in fluconazole-resistant *C. albicans* strains. One of the CdMrr1p mutations, T374I from strain CD57B, corresponds exactly to the T381I mutation that was recently identified in CaMrr1p of an *MDR1*-overexpressing *C. albicans* strain. Similarly, three additional gain-of-function mutations in CdMrr1p, the C866Y mutation from the clinical isolate CM2 and the in-frame deletions T985Δ and [D987-I998]Δ from strains CD51-IIA and CD51-IIB, respectively, are located in two other hot-spot regions where mutations frequently occur in CaMrr1p. In contrast, the S595Y mutation found in strain CD57A is located in a novel region where no gain-of-function mutation has previously been found in Mrr1p. All amino acids that are affected by gain-of-function mutations are identical in CaMrr1p and CdMrr1p (see Fig. S1 in the supplemental material). Except for the S595Y mutation in CdMrr1p, all gain-of-function mutations are located outside of domains predicted by sequence analysis, the Zn_2Cys_6 binuclear cluster DNA-binding domain and a fungus-specific transcription factor domain. The knowledge gained in the present and previous studies about the nature and location of activating

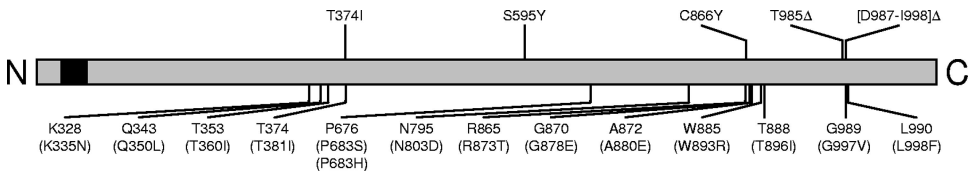

FIG. 4. Location of the gain-of-function mutations identified in CdMrr1p. The CdMrr1p protein is represented as a linear bar. The DNA-binding domain at the N terminus is indicated by black shading. The five mutations found in fluconazole-resistant *C. dubliniensis* strains in the present study are shown above the bar. Positions at which gain-of-function mutations have previously been identified in fluconazole-resistant *C. albicans* strains are indicated below the bar, and the corresponding positions in CaMrr1p are given in parentheses.

mutations in Mrr1p will be highly useful, once other functional domains of this transcription factor have been experimentally defined.

CdMrr1p is able to activate the *MDR1* promoter in the heterologous species *C. albicans*, suggesting that CaMrr1p and CdMrr1p recognize the same binding site, which should be present in the *MDR1* promoters of both species. The Ca*MDR1* and Cd*MDR1* upstream regions are 77% identical within 1 kb before the start codons, with many stretches of complete or nearly complete identity (see Fig. S2 in the supplemental material). The binding sites reported for the transcription factors Cap1p and Mcm1p, which have also been implicated in the regulation of Ca*MDR1* expression, are not completely conserved in the Cd*MDR1* upstream region (27, 28). For a detailed understanding of how Mrr1p activates the expression of *MDR1*, it will be important to determine the binding site of this transcription factor in the *MDR1* promoter and its other target genes, which is currently a major goal in our laboratories.

Most *MDR1*-overexpressing *C. albicans* strains have become homozygous for mutated *MRR1* alleles, and deletion of one of the alleles results in decreased levels of *MDR1* expression and a partial loss of drug resistance, demonstrating that the loss of heterozygosity further increases drug resistance, once a gain-of-function mutation in *MRR1* has occurred (5, 21). A similar observation was made in the present study with the five *MDR1*-overexpressing *C. dubliniensis* strains, in which only the mutated *MRR1* alleles, but no wild-type alleles, were found. For the three susceptible parental strains and the five resistant strains derived from them, all sequenced clones that were obtained from independent PCRs were identical, and no polymorphic positions were found in the regions analyzed by direct sequencing of the PCR products, suggesting that each strain contained two identical *MRR1* alleles. Although we cannot exclude the (unlikely) possibility that both the resistant strains and their susceptible progenitors contain a second *MRR1* allele that was not amplified with the primers used, these results strongly suggest that the presence of fluconazole rapidly selected for the loss of heterozygosity in strains that had acquired a gain-of-function mutation in *MRR1*, as was previously shown for *C. albicans*.

Another interesting aspect of the present investigation is the finding that two fluconazole-resistant strains (strains CD51-IIA and CD51-IIB) that were independently generated in vitro from the same parental strain (strain CD51-II) contained small in-frame deletions in *MRR1* instead of single nucleotide substitutions, which were found in all other *MDR1*-overexpressing *C. albicans* and *C. dubliniensis* strains. A similar observation was recently made for two *C. albicans* strains that overexpressed the ABC transporters *CDR1* and *CDR2* and that contained activating in-frame deletions in the transcription factor *TAC1*, which regulates *CDR1* and *CDR2* expression (2). These findings raise the possibility that strains of both species may differ in the mechanisms used to create genetic diversity. Such strains will be a valuable resource for studies addressing the molecular basis of genetic adaptation mechanisms in these human fungal pathogens.

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REFERENCES

1. **Angiolella, L., A. R. Stringaro, F. De Bernardis, B. Posteraro, M. Bonito, L. Toccacieli, A. Torosantucci, M. Colone, M. Sanguinetti, A. Cassone, and A. T. Palamara.** 2008. Increase of virulence and its phenotypic traits in drug-resistant strains of *Candida albicans*. Antimicrob. Agents Chemother. **52:**927–936.
2. **Coste, A., A. Selmecki, A. Forche, D. Diogo, M. E. Bougnoux, C. d'Enfert, J. Berman, and D. Sanglard.** 2007. Genotypic evolution of azole resistance mechanisms in sequential *Candida albicans* isolates. Eukaryot. Cell **6:**1889–1904.
3. **Cowen, L. E., A. Nantel, M. S. Whiteway, D. Y. Thomas, D. C. Tessier, L. M. Kohn, and J. B. Anderson.** 2002. Population genomics of drug resistance in *Candida albicans*. Proc. Natl. Acad. Sci. USA **99:**9284–9289.
4. **Cowen, L. E., D. Sanglard, D. Calabrese, C. Sirjusingh, J. B. Anderson, and L. M. Kohn.** 2000. Evolution of drug resistance in experimental populations of *Candida albicans*. J. Bacteriol. **182:**1515–1522.
5. **Dunkel, N., J. Blaß, P. D. Rogers, and J. Morschhäuser.** 2008. Mutations in the multidrug resistance regulator *MRR1*, followed by loss of heterozygosity, are the main cause of *MDR1* overexpression in fluconazole-resistant *Candida albicans* strains. Mol. Microbiol. **69:**827–840.
6. **Fekete-Forgacs, K., L. Gyure, and B. Lenkey.** 2000. Changes of virulence factors accompanying the phenomenon of induced fluconazole resistance in *Candida albicans*. Mycoses **43:**273–279.
7. **Gillum, A. M., E. Y. Tsay, and D. R. Kirsch.** 1984. Isolation of the *Candida albicans* gene for orotidine-5′-phosphate decarboxylase by complementation of *S. cerevisiae ura3* and *E. coli pyrF* mutations. Mol. Gen. Genet. **198:**179–182.
8. **Gupta, V., A. Kohli, S. Krishnamurthy, N. Puri, S. A. Aalamgeer, S. Panwar, and R. Prasad.** 1998. Identification of polymorphic mutant alleles of *CaMDR1*, a major facilitator of *Candida albicans* which confers multidrug resistance, and its in vitro transcriptional activation. Curr. Genet. **34:**192–199.
9. **Harry, J. B., B. G. Oliver, J. L. Song, P. M. Silver, J. T. Little, J. Choiniere, and T. C. White.** 2005. Drug-induced regulation of the *MDR1* promoter in *Candida albicans*. Antimicrob. Agents Chemother. **49:**2785–2792.
10. **Karababa, M., A. T. Coste, B. Rognon, J. Bille, and D. Sanglard.** 2004. Comparison of gene expression profiles of *Candida albicans* azole-resistant clinical isolates and laboratory strains exposed to drugs inducing multidrug transporters. Antimicrob. Agents Chemother. **48:**3064–3079.
11. **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:**3007–3012.
12. **Köhler, G. A., T. C. White, and N. Agabian.** 1997. Overexpression of a cloned IMP dehydrogenase gene of *Candida albicans* confers resistance to the specific inhibitor mycophenolic acid. J. Bacteriol. **179:**2331–2338.
13. **Martinez, M., J. L. Lopez-Ribot, W. R. Kirkpatrick, B. J. Coco, S. P. Bachmann, and T. F. Patterson.** 2002. Replacement of *Candida albicans* with *C. dubliniensis* in human immunodeficiency virus-infected patients with oropharyngeal candidiasis treated with fluconazole. J. Clin. Microbiol. **40:**3135–3139.
14. **Millon, L., A. Manteaux, G. Reboux, C. Drobacheff, M. Monod, T. Barale, and Y. Michel-Briand.** 1994. Fluconazole-resistant recurrent oral candidiasis in human immunodeficiency virus-positive patients: persistence of *Candida albicans* strains with the same genotype. J. Clin. Microbiol. **32:**1115–1118.
15. **Moran, G., C. Stokes, S. Thewes, B. Hube, D. C. Coleman, and D. Sullivan.** 2004. Comparative genomics using *Candida albicans* DNA microarrays reveals absence and divergence of virulence-associated genes in *Candida dubliniensis*. Microbiology **150:**3363–3382.
16. **Moran, G., D. Sullivan, J. Morschhäuser, and D. Coleman.** 2002. The *Candida dubliniensis* Cd*CDR1* gene is not essential for fluconazole resistance. Antimicrob. Agents Chemother. **46:**2829–2841.
17. **Moran, G. P., D. M. MacCallum, M. J. Spiering, D. C. Coleman, and D. J. Sullivan.** 2007. Differential regulation of the transcriptional repressor *NRG1* accounts for altered host-cell interactions in *Candida albicans* and *Candida dubliniensis*. Mol. Microbiol. **66:**915–929.
18. **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:**1819–1830.
19. **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 sus-

ceptibilities 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:**617–623.
20. **Morschhäuser, J.** 2002. The genetic basis of fluconazole resistance development in *Candida albicans*. Biochim. Biophys. Acta **1587:**240–248.
21. **Morschhäuser, J., K. S. Barker, T. T. Liu, J. Blaß-Warmuth, R. Homayouni, and P. D. Rogers.** 2007. The transcription factor Mrr1p controls expression of the *MDR1* efflux pump and mediates multidrug resistance in *Candida albicans*. PLoS Pathog. **3:**e164.
22. **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:**1695–1703.
23. **Perea, S., and T. F. Patterson.** 2002. Antifungal resistance in pathogenic fungi. Clin. Infect. Dis. **35:**1073–1080.
24. **Pinjon, E., C. J. Jackson, S. L. Kelly, D. Sanglard, G. Moran, D. C. Coleman, and D. J. Sullivan.** 2005. Reduced azole susceptibility in genotype 3 *Candida dubliniensis* isolates associated with increased Cd*CDR1* and Cd*CDR2* expression. Antimicrob. Agents Chemother. **49:**1312–1318.
25. **Reuß, O., and J. Morschhäuser.** 2006. A family of oligopeptide transporters is required for growth of *Candida albicans* on proteins. Mol. Microbiol. **60:**795–812.
26. **Reuß, O., Å. Vik, R. Kolter, and J. Morschhäuser.** 2004. The *SAT1* flipper, an optimized tool for gene disruption in *Candida albicans*. Gene **341:**119–127.
27. **Riggle, P. J., and C. A. Kumamoto.** 2006. Transcriptional regulation of *MDR1*, encoding a drug efflux determinant, in fluconazole-resistant *Candida albicans* strains through an Mcm1p binding site. Eukaryot. Cell **5:**1957–1968.
28. **Rognon, B., Z. Kozovska, A. T. Coste, G. Pardini, and D. Sanglard.** 2006. Identification of promoter elements responsible for the regulation of *MDR1* from *Candida albicans*, a major facilitator transporter involved in azole resistance. Microbiology **152:**3701–3722.
29. **Ruhnke, M., A. Eigler, I. Tennagen, B. Geiseler, E. Engelmann, and M. Trautmann.** 1994. Emergence of fluconazole-resistant strains of *Candida albicans* in patients with recurrent oropharyngeal candidosis and human immunodeficiency virus infection. J. Clin. Microbiol. **32:**2092–2098.
30. **Ruhnke, M., A. Schmidt-Westhausen, and J. Morschhäuser.** 2000. Development of simultaneous resistance to fluconazole in *Candida albicans* and *Candida dubliniensis* in a patient with AIDS. J. Antimicrob. Chemother. **46:**291–295.
31. **Sanglard, D.** 2002. Resistance of human fungal pathogens to antifungal drugs. Curr. Opin. Microbiol. **5:**379–385.
32. **Staib, P., G. P. Moran, D. J. Sullivan, D. C. Coleman, and J. Morschhäuser.** 2001. Isogenic strain construction and gene targeting in *Candida dubliniensis*. J. Bacteriol. **183:**2859–2865.
33. **Staib, P., and J. Morschhäuser.** 2005. Differential expression of the *NRG1* repressor controls species-specific regulation of chlamydospore development in *Candida albicans* and *Candida dubliniensis*. Mol. Microbiol. **55:**637–652.
34. **Sullivan, D., and D. Coleman.** 1998. *Candida dubliniensis*: characteristics and identification. J. Clin. Microbiol. **36:**329–334.
35. **Sullivan, D. J., G. P. Moran, and D. C. Coleman.** 2005. *Candida dubliniensis*: ten years on. FEMS Microbiol. Lett. **253:**9–17.
36. **Sullivan, D. J., G. P. Moran, E. Pinjon, A. Al-Mosaid, C. Stokes, C. Vaughan, and D. C. Coleman.** 2004. Comparison of the epidemiology, drug resistance mechanisms, and virulence of *Candida dubliniensis* and *Candida albicans*. FEMS Yeast Res. **4:**369–376.
37. **Sullivan, D. J., T. J. Westerneng, K. A. Haynes, D. E. Bennett, and D. C. Coleman.** 1995. *Candida dubliniensis* sp. nov.: phenotypic and molecular characterization of a novel species associated with oral candidosis in HIV-infected individuals. Microbiology **141**(Pt 7)**:**1507–1521.
38. **Wirsching, S., G. P. Moran, D. J. Sullivan, D. C. Coleman, and J. Morschhäuser.** 2001. *MDR1*-mediated drug resistance in *Candida dubliniensis*. Antimicrob. Agents Chemother. **45:**3416–3421.
39. **Yan, L., J. D. Zhang, Y. B. Cao, P. H. Gao, and Y. Y. Jiang.** 2007. Proteomic analysis reveals a metabolism shift in a laboratory fluconazole-resistant *Candida albicans* strain. J. Proteome Res. **6:**2248–2256.