Overexpression of *Candida albicans CDR1, CDR2,* or *MDR1* Does Not Produce Significant Changes in Echinocandin Susceptibility†

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The micafungin and caspofungin susceptibilities of *Candida albicans* laboratory and clinical isolates and of *Saccharomyces cerevisiae* strains stably hyperexpressing fungal ATP-binding cassette (ABC) or major facilitator superfamily (MFS) transporters involved in azole resistance were determined using three separate methods. Yeast strains hyperexpressing individual alleles of ABC transporters or an MFS transporter from *C. albicans* gave the expected resistance profiles for the azoles fluconazole, itraconazole, and voriconazole. The strains hyperexpressing *CDR2* showed slightly decreased susceptibility to caspofungin in agar plate drug resistance assays, as previously reported, but increased susceptibility to micafungin compared with either the strains hyperexpressing *CDR1* or the null parent deleted of seven ABC transporters. The strains hyperexpressing *CDR1* showed slightly decreased susceptibility to micafungin in these assays. A *C. albicans* clinical isolate overexpressing both Cdr1p and Cdr2p relative to its azole-sensitive isogenic progenitor acquired resistance to azole drugs and showed reduced susceptibility to caspofungin and slightly increased susceptibility to micafungin in agar plate drug resistance assays. None of the strains showed significant resistance to micafungin or caspofungin in liquid microdilution susceptibility assays. The antifungal activities of micafungin and caspofungin were similar in agarose diffusion assays, although the shape and size of the caspofungin inhibitory zones were affected by medium composition. The assessment of micafungin and caspofungin potency is therefore assay dependent; the differences seen with agar plate drug resistance assays occur over narrow ranges of echinocandin concentrations and are not of clinical significance.

Systemic fungal infection is a problem of increasing clinical significance for the immunocompromised, especially organ transplant recipients and cancer patients. Until recently only three classes of antifungal drugs were available to treat people with these infections: the polyene antibiotics (e.g., amphotericin B), the azole drugs (imidazoles and triazoles), and fluoropyrimidines (flucytosine). The candins, such as caspofungin (CSF) and micafungin (MCF), are members of a novel class of antifungals. They inhibit glucan synthase activity and the synthesis of the essential cell wall component β-1,3-glucan, and they provide new therapeutic options for the treatment of systemic *Aspergillus* and *Candida* infections (6). CSF (Cancidas; Merck), an artificial derivative of echinocandin (12), was the first candin to gain FDA approval. MCF (Mycamine; Fujisawa Pharmaceutical Co., now Astellas Pharma Inc.) is a new semisynthetic echinocandin derived from a lead compound obtained from the soil organism *Coleophoma empetri* (34). Both candins are effective against medically important yeasts and filamentous fungi including *Aspergillus fumigatus, Candida albicans,* non-*C. albicans Candida* species, and azole-resistant *Candida* species (3, 6, 28, 33). Their antifungal spectrum is limited, however, because some clinically important fungal species, including *Cryp-*

tococcus neoformans, Trichosporon spp., and *Fusarium* spp., are not susceptible and the zygomycetes (*Mucor, Rhizopus,* or *Absidia*) appear innately resistant (10, 35). Despite the ready selection in vitro of candin-resistant *Saccharomyces cerevisiae* (7) and *C. albicans* (13) variants with mutations in the catalytic subunit of glucan synthase (27), reports of clinical *Candida* isolates with acquired candin resistance are rare. Because the candins are not structurally or functionally related to the polyene or azole drugs, cross-resistance to these agents was expected to be infrequent. However, clinical isolates that are resistant to both candins and azoles have been reported for *C. albicans* (9) and *Candida parapsilosis* (20). The overexpression of ABC transporters in azole-resistant cells is a concern because they confer resistance to a wide range of structurally unrelated xenobiotics. It was therefore important to determine whether candin susceptibility could be affected by the overexpression of such transporters. Schuetzer-Muehlbauer et al. (30) used MIC determinations and agar plate resistance assays for *S. cerevisiae* and *C. albicans* cells overexpressing plasmid-borne copies of *C. albicans CDR1* and *CDR2* to suggest that Cdr2p confers CSF resistance. In contrast, studies of mutant laboratory strains (5) and clinical isolates (3) concluded that CSF was not a substrate of *C. albicans* ABC transporters. However, individual efflux pumps that are stably expressed have yet to be fully analyzed for their abilities to pump candins.

We have used a *Saccharomyces cerevisiae* heterologous membrane protein hyperexpression system to construct a panel of mutant strains that hyperexpress individual transporter alleles cloned from fungal pathogens (19, 23, 25). The panel

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TABLE 1. Yeast strains used in this study

Species and strain	Source	Reference
Candida albicans		
ATCC 10261	American Type Culture Collection, Manassas, Va.	
TIMM3163	Clinical isolate, resistant to azoles, from Teikyo University (Tokyo, Japan)	15
SC5314	*Candida albicans* database strain	11
TL1	Clinical isolate, sensitive to azoles, obtained from T. White (Seattle, WA)	17
TL3	Clinical isolate, resistant to azoles, obtained from T. White (Seattle, WA)	17
Candida glabrata CBS138	Centraalbureau voor Schimmelcultures, Utrecht, The Netherlands	
Cryptococcus neoformans CDC551	Serotype A, sexual type α, from Chiba University (Chiba, Japan)	

allows functional analysis of membrane transporters that confer resistance to xenobiotics by using simple drug susceptibility and biochemical assays (21, 36, 37). Here we demonstrate that the functional hyperexpression of single fungal transporters in *S. cerevisiae* confers detectable candin-specific susceptibility changes in agar plate drug resistance assays. These effects are unlikely to involve the transport activity of known azole efflux pumps or to have clinical significance, because they are not reflected in other assays of drug susceptibility, including those with azole-resistant clinical isolates of *C. albicans*.

MATERIALS AND METHODS

Yeast strains and culture conditions. The yeast strains used in this study are listed in Table 1 (see also Table S1 in the supplemental material). *S. cerevisiae* cells were routinely maintained on CSM-Ura (complete synthetic medium without uracil) agar plates (22). Uridine (50 μg/ml) was added to CSM-Ura for growth of the AD1-8u⁻ host. *C. albicans* cells were routinely maintained on yeast extract-peptone-dextrose (YPD) agar plates (22).

Plasmid construction and yeast transformation. KOD DNA polymerase (Toyobo, Osaka, Japan) was used to PCR amplify the open reading frames (ORFs) of fungal ABC transporters or major facilitator superfamily (MFS) transporters (*C. albicans CDR1, CDR2,* and *MDR1, Candida glabrata CDR1,* and *C. neoformans MDR1*) from genomic DNAs of the strains listed in Table 1. The ORFs were directionally cloned into PacI and NotI sites of the pABC3 vector (18). *S. cerevisiae* AD1-8u⁻ cells (21) were transformed by the lithium acetate method (Alkali-Cation Yeast Transformation kit; Bio 101, Irvine, CA) using a transformation cassette from pABC3 that contained the ORF. Ura⁺ transformants with the ORF integrated into the *PDR5* chromosomal locus were selected on CSM-Ura plates and tested for fluconazole (FLC) resistance on FLC-containing CSM-Ura plates. The cloned genes from Ura⁺ Flc^R transformants were PCR amplified from genomic DNA and their sequences confirmed.

Compounds. MCF and CSF were synthesized by Fujisawa Pharmaceutical Co., Ltd. (Osaka, Japan). Voriconazole (VRC) was synthesized by the NARD Institute, Ltd. (Hyogo, Japan). Itraconazole (ITC) was purchased from Janssen-Kyowa (Tokyo, Japan). FLC (Diflucan; aqueous solution) was purchased from Pfizer Laboratories Limited (Auckland, New Zealand), and nystatin (NYT) was from Sigma (St. Louis, Mo.). MCF and CSF were dissolved in sterile distilled water, and the other compounds were dissolved in dimethyl sulfoxide.

Preparation of plasma membrane fractions and protein identification. Plasma membrane fractions were isolated from the *S. cerevisiae* strains as described by Niimi et al. (22). The expression of ABC proteins (140 to 170 kDa) and MFS protein (60 kDa) was detected as Coomassie blue R250-stained bands with the predicted molecular masses after separation by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) in gels containing 8% acrylamide. The identity of each protein band was confirmed by either immunodetection (21) or mass spectrometry tryptic fingerprint analysis (24). The Cdr1p- and Cdr2p-specific polyclonal antibodies were kind gifts from Dominique Sanglard and Martine Raymond, respectively.

Drug susceptibility assays. (i) Agar plate drug resistance assays. Assays were performed as previously described (30). Cell growth was monitored after incubation at 30°C for 48 h.

(ii) MIC determination by liquid microdilution susceptibility assay and checkerboard drug competition assay. Antifungal MICs were measured using 96-well microtiter plates and CSM (pH 7.0) buffered with 10 mM morpholineethanesulfonic acid and 20 mM HEPES instead of RPMI (22). MICs for *S. cerevisiae*

and *C. albicans* strains were determined after 48 h of incubation at 30°C and 24 h of incubation at 35°C, respectively. Each MIC determination was performed in triplicate in at least three independent experiments. Cell growth was monitored at 590 nm using an EL340 Bio Kinetics plate reader (BioTek Instruments). The MICs of MCF and CSF were the concentrations giving >95% growth inhibition (MIC₉₅), while for azoles and NYT the MICs were the concentrations giving >80% and 100% growth inhibition, respectively, compared with the no-drug control. The viability of each strain at and above the MIC was tested by spotting 5-μl samples from microtiter wells onto drug-free YPD agar and incubating at 30°C for 48 h. The minimum fungicidal concentration (MFC) was defined as the lowest concentration of drug that gave no viable colonies on YPD. The checkerboard drug susceptibility assay using candins and the drug efflux pump substrate FLC was performed as previously described (22).

(iii) Drug diffusion assays. The susceptibilities of yeast strains to candin and azole drugs were compared using diffusion assays in either YPD agar plates with a reduced agar concentration (0.8%) in the overlay, YPD agarose (with 0.6% agarose instead of the agar) plates with 0.4% agarose in the overlay, CSM agar plates with 0.8% agar in the overlay, or CSM agarose (with 0.6% agarose) plates with 0.4% agarose in the overlay. The plates were seeded with 5 × 10⁵ cells in 5 ml of overlay medium. The indicated amount of each drug was applied to individual sterile paper disks and placed on the overlay. Cell growth was monitored after incubation at 30°C for 48 h for *S. cerevisiae* strains and after 24 h for *C. albicans* strains.

RESULTS

Overexpression of drug efflux pumps in plasma membrane fractions of *S. cerevisiae*. The *PDR5* gene from *S. cerevisiae*, the two alleles (A and B) of *CDR1* and of *CDR2* from the azole-sensitive *C. albicans* laboratory strain ATCC 10261, and the A allele of *CDR2* from the *C. albicans* database strain SC5314 were cloned into the *PDR5* locus of the hypersensitive *S. cerevisiae* AD1-8u⁻ host. The construction and analysis of the AD/MDR1 strain (expressing MFS transporter Mdr1p [also called Ben^rp]) are described elsewhere (25). Yeast strains were also constructed that hyperexpressed the A or B alleles of *CDR2* from three clinical isolates obtained from AIDS patients: azole-resistant TIMM3163 (15) and a pair of isogenic strains (the azole-sensitive parent TL1 and the azole-resistant daughter TL3) that were sequentially isolated from another patient during azole treatment (Tables 1 and 2) (17). Allelic variation is absent in the 30-kb region of the SC5314 genome that includes both *CDR1* and *CDR2* (2, 11).

Coomassie blue-stained SDS-PAGE profiles of plasma membrane fractions from representative AD/CDR1, AD/CDR2, and AD/PDR5 strains revealed a 170-kDa protein band that was not present in the parental *S. cerevisiae* host AD1-8u⁻. The identity of the 170-kDa band was confirmed by Western blot analysis using Cdr1p- and Cdr2p-specific antibodies or by mass spectrometry tryptic fingerprint analysis. The levels of expression of pairs of alleles from either laboratory strains or clinical *C. albicans* isolates were comparable. The AD/MDR1 strain pro-

TABLE 2. MICs of micafungin and other drugs for *S. cerevisiae* strains hyperexpressing fungal transporters and for *C. albicans* strains[a]

Strain	MIC (μg/ml) of:					
	MCF	CSF	FLC	ITC	VRC	NYT
S. cerevisiae						
AD1-8u⁻	0.125	1	0.5	0.031	0.063	2
AD/CaMDR1	0.125	1	60	0.031	0.5	2
AD/CaCDR1 A	0.125	1	200	>32	16	2
AD/CaCDR1 B	0.250	1	300	>32	16	2
AD/CaCDR2 A	0.125	1	80	>32	4	2
AD/CaCDR2 B	0.125	2	160	>32	4	2
AD/CgCDR1	0.125	1	300	>32	4	4
AD/CnMDR1	0.125	1	5	>32	0.063	2
C. albicans						
ATCC 10261	0.0078	0.25	0.5	0.063	0.063	8
TIMM3163	0.0156	0.25	32	0.5	0.5	8
TL1	0.0078	0.5	1	0.125	0.063	8
TL3	0.0156	0.5	32	0.5	0.5	8

[a] Cells were incubated in CSM (pH 7.0) at 30°C for 48 h for *S. cerevisiae* and at 35°C for 24 h for *C. albicans* strains. *C. albicans* *CDR1*, *CDR2*, and *MDR1* were from ATCC 10261. Three separate experiments with triplicate determinations each gave identical end points.

duced an immunologically identifiable 55-kDa protein band (25). The AD/CDR2 constructs gave consistently lower Pma1p expression than the AD/CDR1 and AD/PDR5 constructs.

Agar plate drug resistance assays. To investigate whether stable hyperexpression of a single genomic copy of *CDR2* conferred resistance to MCF and to CSF, as described by Schuetzer-Muehlbauer et al. for plasmid-encoded *CDR2* (30), the agar plate drug resistance assay was used for *S. cerevisiae* strains that hyperexpressed the *C. albicans* ATCC 10261 *CDR2* A or B alleles. The parental strain AD1-8u⁻ and derivative strains transformed with either the empty pABC3 transformation cassette, the *CDR1* A or B alleles, or *MDR1* (all from ATCC 10261) were used as controls. Each strain was tested using CSF and MCF on YPD plates. Some *S. cerevisiae* cells hyperexpressing *CDR2* survived on YPD agar plates containing ≤0.5 but not 1 μg/ml CSF, while cells of the parental strain (AD1-8u⁻), AD/pABC3, and those expressing *CDR1* or *MDR1* were fully susceptible to CSF at 0.3 to 0.5 μg/ml (Fig. 1). In contrast, the AD/CDR2 cells were susceptible to MCF (Fig. 1). The growth of cells expressing either the A or the B allele of *CDR2* was inhibited at 0.08 μg/ml MCF, and growth was abolished at 0.1 μg/ml MCF, whereas the parental strain and the other control strains grew at both concentrations.

The CSF and MCF susceptibilities of *S. cerevisiae* strains expressing *CDR2* alleles from a variety of azole-resistant and -sensitive *C. albicans* strains were measured (see Fig. S1 in the supplemental material). The growth of the control strain AD/pABC3 was abolished by CSF at 0.2 μg/ml, but colonies of all the strains hyperexpressing *CDR2* survived on YPD agar containing ≤0.6 μg/ml CSF. In contrast, each AD/CDR2 construct was more susceptible to 0.1 μg/ml MCF than the AD/pABC3 control, and no cells survived on agar containing 0.2 μg/ml MCF (see Fig. S1 in the supplemental material).

In order to determine whether elevated expression of drug efflux pumps, in particular Cdr2p, is associated with reduced candin susceptibility in *C. albicans*, we performed agar plate

drug resistance assays for candins on YPD agar with the *C. albicans* strains from which the *CDR2* alleles were cloned (Table 1; see also Table S1 in the supplemental material). We previously showed that strains TL3 and TIMM3163 had elevated plasma membrane expression of the Cdr1p and Cdr2p efflux pumps (as determined by Western blot analysis [15] [A. R. Holmes, unpublished results]), and increased energy-dependent efflux of the Cdr substrate R6G, relative to the laboratory strain ATCC 10261 and TL1 (unpublished results). TIMM3163 was highly susceptible to both candins in agar plate drug resistance assays, whereas the other strains survived 0.25 μg/ml CSF and 0.063 μg/ml MCF. There were subtle differences in susceptibility to CSF and MCF between TL1 and TL3. The drug pump-overexpressing daughter strain (TL3) survived 0.5 μg/ml CSF, while the parental strain, TL1, did not. In contrast, TL1 grew slightly better than TL3 at 0.25 μg/ml MCF (Fig. 2A). However, these effects occurred within narrow ranges of candin concentrations. Identical results with agar plate drug resistance assays were obtained in at least three replicate experiments.

MICs of azoles and candins for yeast strains hyperexpressing transporters. The significance of the reduced susceptibilities of strains hyperexpressing *CDR1* and *CDR2* to MCF and CSF, respectively, and the enhanced susceptibility of the strain hyperexpressing *CDR2* to MCF in agar plate drug resistance assays were assessed by determining the liquid MICs of MCF, CSF, and azoles for the panel of *S. cerevisiae* mutants (see Table S1 in the supplemental material). CSM (pH 7.0) was used for MIC determinations (22) because RPMI medium did not support the growth of the *S. cerevisiae* strains. The MCF and CSF MICs for the parent strain, AD1-8u⁻, and for cells hyperexpressing drug efflux pumps were all 0.125 to 0.25 μg/ml and 1 to 2 μg/ml, respectively (Table 2). The MFCs of both candins for all the test strains and the parent strain, AD1-8u⁻, were at least 8- to 16-fold higher than their MICs. Interstrain differences in MIC profiles at sub-MIC drug concentrations were not detected, and all yeast strains gave the same MIC₉₅ (data not shown). As expected, the strains hyperexpressing ABC pumps had MICs for the three azoles that were significantly higher than those for the parental strain, AD1-8u⁻, while susceptibilities to NYT were not altered. Hyperexpression of Ca*MDR1* (MFS pump) conferred resistance to FLC and, to a lesser extent, to VRC. Compared with its orthologs, overexpression of the *C. neoformans* ABC transporter Mdr1p in *S. cerevisiae* conferred a different resistance to azole drugs, but this strain was just as susceptible to MFC and CSF as the other strains (Table 2).

The candin MICs were also determined for the *C. albicans* strains by using CSM (pH 7.0). All the strains had MCF and CSF MICs within the ranges of 0.0078 to 0.0156 μg/ml and 0.25 to 0.5 μg/ml, respectively (Table 2).

Drug diffusion susceptibility assays. Drug diffusion susceptibility assays were performed using *S. cerevisiae* strains hyperexpressing *C. albicans* *CDR1* A and B alleles, the *CDR2* A and B alleles, Ca*MDR1*, the *C. glabrata* transporter Cg*CDR1*, and *C. neoformans* Cn*MDR1*. The sizes of inhibitory zones obtained for CSF on media solidified with agar were significantly smaller than those on media containing agarose (data not shown). Agarose was therefore used instead of agar to enhance the diffusion of the test compounds, especially hydrophobic agents (22). As expected,
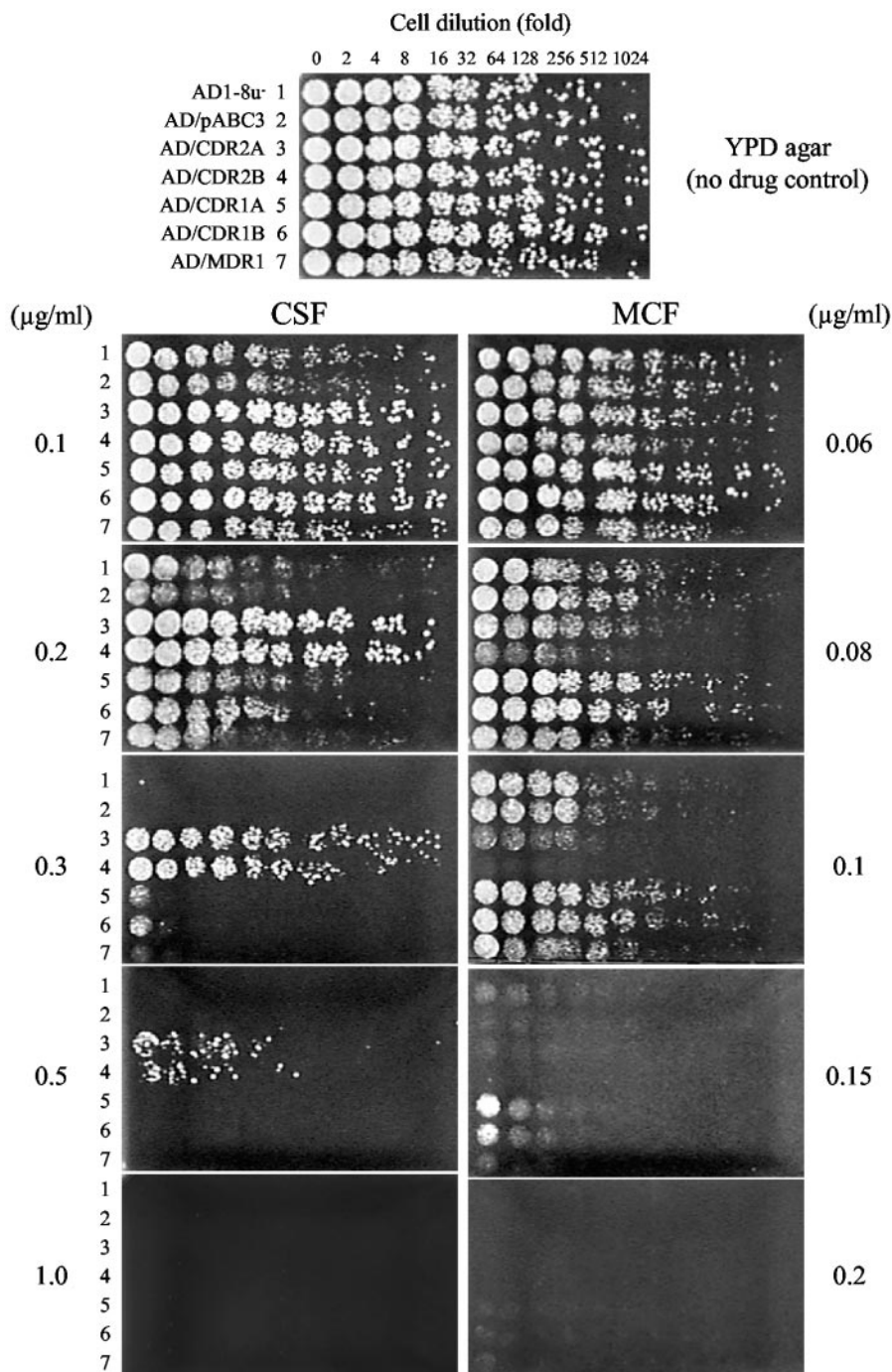
FIG. 1. CSF and MCF susceptibilities of *S. cerevisiae* cells hyperexpressing *C. albicans* drug efflux pumps in agar plate assays. Alleles were cloned from *C. albicans* ATCC 10261. Twofold serial dilutions of exponentially growing cells (5 μl) were spotted onto YPD agar medium containing CSF or MCF at the indicated concentrations and incubated at 30°C for 48 h as described in Materials and Methods. Representative results of an experiment carried out three times are shown.

the parent strain AD1-8u⁻ was sensitive to all three azoles and both candins, while strains hyperexpressing ABC pumps (except Cn*MDR1*) were resistant to all azoles but were sensitive to both candins on both YPD (Fig. 3) and CSM (see Fig. S2 in the supplemental material). There was no difference in sensitivity between cells expressing different *C. albicans* alleles of *CDR1* or *CDR2*, and all the strains appeared slightly less susceptible to CSF

on YPD medium than the parent strain, but the irregular shape of the zones of inhibition made comparison difficult (Fig. 3). This effect was reproducible and medium specific; it was not seen with CSM agarose (see Fig. S2 in the supplemental material). MCF, unlike CSF, gave similar-size circular inhibitory zones independent of medium composition or solidifier. We also performed azole and candin drug diffusion susceptibility assays on YPD
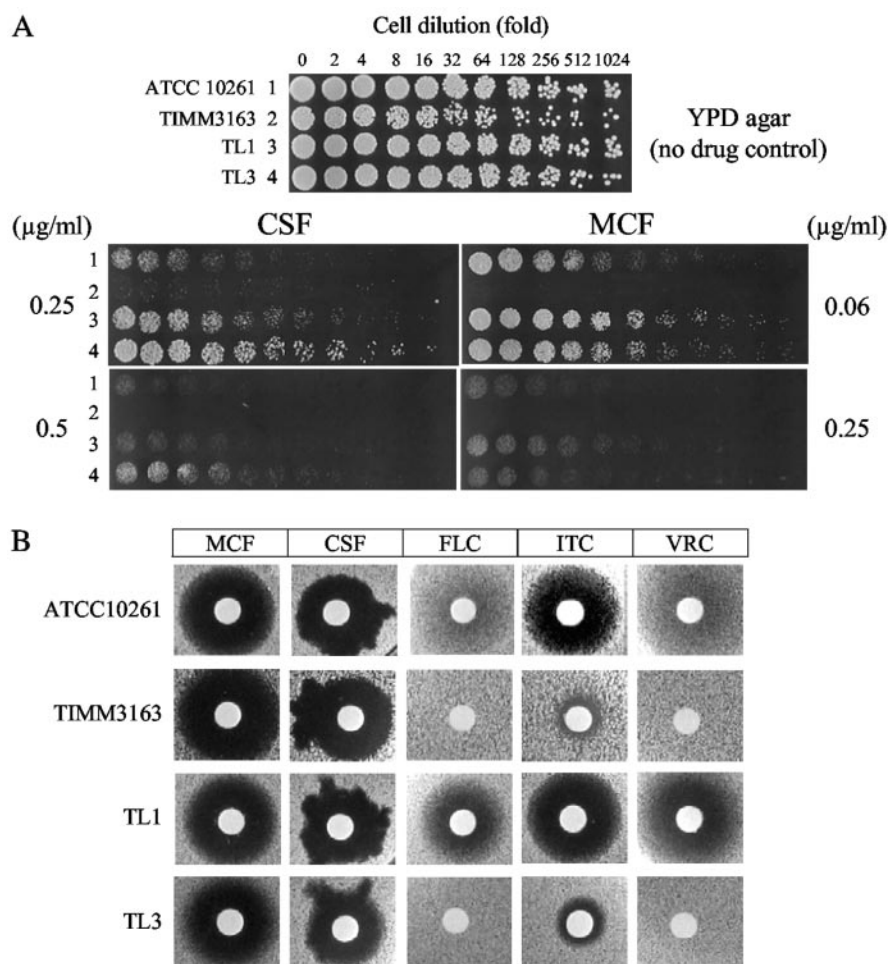
FIG. 2. CSF and MCF susceptibilities of a *C. albicans* laboratory strain and of clinical isolates from which *CDR1* and *CDR2* were cloned. (A) The agar plate resistance assay was performed as for Fig. 1. (B) Susceptibilities of *C. albicans* strains to candins and azoles were determined by a drug diffusion assay on YPD agarose. The following amounts (in micrograms) of drugs were applied to disks: MCF, 2; CSF, 2; FLC, 5; ITC, 0.5; VRC, 0.2.

agarose for the four *C. albicans* strains. Both TL3 and TIMM3163 showed significantly higher resistance to the azoles FLC, ITC, and VRC in the agarose drug diffusion assay than strains ATCC 10261 and TL1, but neither of the azole-resistant strains showed modified susceptibility to either MCF or CSF (Fig. 2B). These results were replicated in three independent experiments.

Checkerboard drug competition assays. Competition between the candins and the known drug efflux pump substrate FLC was tested using checkerboard MIC assays. In the strains hyperexpressing *C. albicans CDR1*, *CDR2*, or *MDR1*, no competition was detected between FLC and either CSF or MCF (data not shown).

DISCUSSION

Schuetzer-Muehlbauer et al. (30) used agar plate drug resistance assays of *S. cerevisiae* and *C. albicans* strains overexpressing plasmid-borne *C. albicans* multidrug efflux pumps to conclude that *CDR2* conferred hyper-resistance to CSF. We used the same assay to show that the stable, functional, genomic hyperexpression of Ca*CDR2* alleles obtained from either laboratory strains

or clinical isolates conferred on *S. cerevisiae* reduced susceptibility to CSF but enhanced susceptibility to MCF, in each case over a narrow concentration range of the candin drug. Furthermore, comparable hyperexpression of Ca*CDR1* alleles conferred on yeast reduced susceptibility to MCF only over an even narrower concentration range. The modified MCF and CSF susceptibilities conferred by hyperexpression of Ca*CDR1* and Ca*CDR2* alleles are unlikely to correlate with pump efflux activity, as was seen with the high-level azole resistance conferred by pump overexpression in yeast (23), or to have clinical significance, because these strains gave liquid MICs and drug diffusion inhibitory zones in CSM that were comparable with those of the null parent. Four *C. albicans* strains were also tested in the three separate susceptibility assays. Two of the strains (TL3 and TIMM3163) were cross resistant to azole drugs and had higher functional expression of Cdr1p and Cdr2p than two azole-sensitive strains (ATCC 10261 and TL1 [15] [A. R. Holmes, unpublished observations]). There were subtle differences between strains TL1 and TL3 in survival on CSF- or MCF-containing agar plates, comparable to the differences seen for the *S. cerevisiae* strains overexpressing drug pumps (Cdr1p and
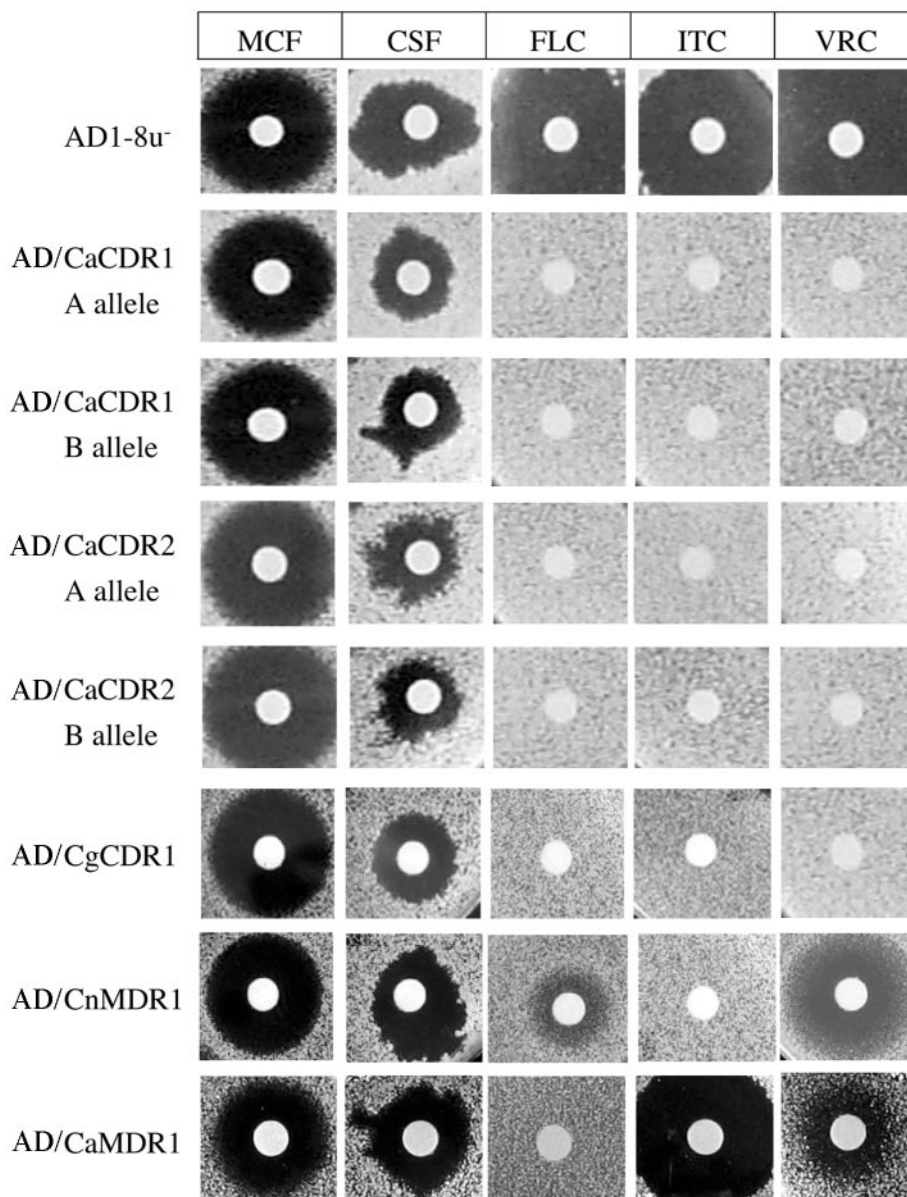
FIG. 3. Susceptibilities of *S. cerevisiae* strains hyperexpressing membrane transporters to candins and azoles were determined by a drug diffusion assay on YPD agarose. Amounts of drugs applied per disk were the same as for Fig. 2B except for CSF (1 µg).

Cdr2p) and the parent AD1-8u⁻, but all four strains were equally susceptible to both MCF and CSF in agarose drug diffusion and liquid microdilution susceptibility assays. These observations, which include an isogenic pair of isolates from the same patient (TL1 and TL3 [17]), confirmed that the increased functional expression of Cdr1p and Cdr2p did not give significant candin resistance in *C. albicans* strains.

Hyperexpression of Cdr2p in the plasma membrane substantially reduced the amount of the essential plasma membrane proton pump expression in membrane fractions, and this effect may cause the reduced growth rates that were observed. Furthermore, Cdr1p and Cdr2p may be phospholipid flippases (31), and energy-independent facilitated diffusion has been implicated in CSF uptake by *C. albicans* at low drug concentrations (~1 µg/ml), but it is not known if the latter transport

mechanism is essential for CSF activity or if MCF uses the same transporter (26). Although the precise roles of Cdr1p and Cdr2p in modifying slightly the susceptibility of yeast to low concentrations of candin antifungals in the agar plate drug resistance assay have yet to be determined, these effects may be related to a time-dependent (time-kill) effect previously detected for MCF with individual *C. albicans* strains (8). Thus, although the agar plate drug resistance assay is reproducibly highly sensitive to minor differences in drug susceptibility over narrow windows of drug concentrations, it should be complemented by other in vitro assay methods in evaluating candin efficacy.

The growth of resistant variants within the test population can cause the trailing effect seen with FLC-resistant or -susceptible, dose-dependent *C. albicans* strains (14). Thus, the

MIC at which 80% of growth is inhibited is commonly used as an end point for azole drugs. The MCF and CSF MIC profiles of the *S. cerevisiae* strains in CSM did not show the trailing effect (data not shown). The MICs of the candins (0.125 to 0.25 μg/ml for MCF and 1 to 2 μg/ml for CSF) were therefore determined at >95% inhibition of cell growth. The surviving colonies in test wells above the MIC accounted for less than 20% of the initial inoculum, and no paradoxical growth effect was observed (32). A subtle difference in the susceptibility to CSF of cells hyperexpressing Cdr1p or Cdr2p was detected in agarose drug diffusion assays using YPD, but not in CSM agarose or in liquid CSM MIC assays. Interpretation of the drug diffusion assay for CSF was complicated by the irregular shape of inhibitory zones on YPD and the effects of medium composition and solidifier on efficacy. In contrast, MCF showed consistent inhibitory activity with all strains tested that was independent of the medium and solidifier. This may be due to the higher water solubility of MCF than of CSF and to the fact that CSF is known to form aggregates in solution at higher concentrations (26). The well diffusion method using Casitone agar medium gives data that agree with those obtained by the NCCLS (now CLSI) M27-A2 method for CSF against *Candida* spp. (16, 21a). The composition of YPD (yeast extract and peptone) may therefore critically affect susceptibility to CSF in solid and liquid media. MCF gave well-correlated agar diffusion and liquid MIC data (by NCCLS M38-A) for *Aspergillus* spp. (1, 21b), but MCF activity for *Candida* spp. using different medium types has yet to be fully assessed. Although NCCLS M27-A2 provides a reference method for testing the activity of candins, the MICs are affected by medium composition (4, 29). The MIC and agarose diffusion data obtained for our yeast constructs correlated well for the azoles but less well for MCF and CSF. A comprehensive set of standardized in vitro susceptibility tests is needed for this new class of antifungals. Resistant variants seen in the inhibitory zones with FLC were not found with either candin, suggesting that development of candin resistance may be infrequent in these *S. cerevisiae* strains.

We have extended the findings of Schuetzer-Muehlbauer et al. (30) for the agar plate drug resistance assay to strains that stably express single alleles encoding some of the most clinically important drug efflux pumps from *C. albicans*, *C. glabrata*, and *C. neoformans* involved in multidrug resistance. MCF and CSF were also effective against *C. albicans* clinical isolates functionally overexpressing fungal efflux pumps in a variety of assays under different growth conditions. Furthermore, checkerboard assays showed that neither MCF nor CSF competes with the pump substrate FLC for the Cdr1p, Cdr2p, or Mdr1p efflux pump from *C. albicans*. We therefore predict that the major drug efflux pumps overexpressed in azole-resistant *C. albicans*, *C. glabrata*, or *C. neoformans* strains are unlikely to cause clinically significant cross-resistance to CSF and MCF.

ACKNOWLEDGMENTS

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