Abc1p Is a Multidrug Efflux Transporter That Tips the Balance in Favor of Innate Azole Resistance in *Candida krusei*[▽]

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Most *Candida krusei* strains are innately resistant to fluconazole (FLC) and can cause breakthrough candidemia in immunocompromised individuals receiving long-term prophylactic FLC treatment. Although the azole drug target, Erg11p, of *C. krusei* has a relatively low affinity for FLC, drug efflux pumps are also believed to be involved in its innate FLC resistance. We describe here the isolation and characterization of Abc1p, a constitutively expressed multidrug efflux pump, and investigate *ERG11* and *ABC1* expression in *C. krusei*. Examination of the *ERG11* promoter revealed a conserved azole responsive element that has been shown to be necessary for the transcription factor Upc2p mediated upregulation by azoles in related yeast. Extensive cloning and sequencing identified three distinct *ERG11* alleles in one of two *C. krusei* strains. Functional overexpression of *ERG11* and *ABC1* in *Saccharomyces cerevisiae* conferred high levels of resistance to azoles and a range of unrelated Abc1p pump substrates, while small molecule inhibitors of Abc1p chemosensitized *C. krusei* to azole antifungals. Our data show that despite the presence of multiple alleles of *ERG11* in some, likely aneuploid, *C. krusei* strains, it is mainly the low affinity of Erg11p for FLC, together with the constitutive but low level of expression of the multidrug efflux pump Abc1p, that are responsible for the innate FLC resistance of *C. krusei*.

Paradoxically, medical advances of the 20th century have led to a significant increase in the incidence of life-threatening fungal infections. Most of the more than 100,000 described fungal species live in the environment and do not colonize humans. However, with the increase in the number of immunocompromised individuals due to the AIDS epidemic, the number of organ transplant patients receiving immunosuppressant treatment, or the number of cancer patients undergoing chemotherapy, an increase in the incidence of fungal infections has ensued (19). Most fungal infections are caused by *Candida* species, with *Candida albicans* predominating, followed by *C. glabrata*, *C. parapsilosis*, *C. tropicalis*, and, in fifth place, *C. krusei* (34). While *C. albicans* and *C. parapsilosis* are common in neonatal and pediatric age groups *C. glabrata* and *C. krusei* infections are becoming more important in the elderly population (33).

A limited choice of antifungal agents, however, is available to the clinician (19). One of the most commonly used class of antifungals is the azoles, which include fluconazole (FLC) and itraconazole (ITC), and the more recently introduced broad-spectrum triazoles voriconazole (VRC) and posaconazole (35), both of which exhibit improved pharmacokinetic properties and a broader spectrum of antifungal activity. The target for the azoles is cytochrome P450 lanosterol 14 α -demethylase, Erg11p, which is also known as Cyp51p, an essential enzyme of the ergosterol biosynthesis pathway (18). FLC is still the most commonly used azole antifungal because it is a well-tolerated drug with few side effects (12). Azoles, however, are fungistatic

rather than fungicidal. This azole tolerance is mediated by the Hsp90-dependent calcineurin stress response pathway (3, 5, 6). It allows fungal cells to develop resistance at high frequency using a range of mechanisms. These processes are well documented and include (i) the mutation or the overexpression of the drug target Erg11p leading to medium levels of resistance, (ii) mutations in enzymes of the ergosterol biosynthesis pathway (e.g., *ERG3*) that compensate for the accumulation of toxic ergosterol intermediates in cells treated with azoles, and (iii) the overexpression of drug efflux pumps that leads to the highest levels of resistance (1, 2, 19, 55).

Drug efflux pumps belong either to the ATP-binding cassette (ABC) family of transporters or the major facilitator superfamily of transporters. These pumps are present in all living organisms and are often involved in protecting cells from the harmful effects of toxic compounds. Their overexpression is associated with the multidrug-resistant phenotype of clinically important pathogenic microorganisms. Although most *Candida* species are susceptible to azole antifungals, *C. krusei* and *C. glabrata* are two *Candida* species that are often naturally resistant to FLC (25, 34, 35, 38). The two FLC-inducible ABC transporters CgCdr1p and CgPdh1p, whose upregulation is mediated by the transcription factor CgPdr1p, are the main cause for the innate FLC resistance of *C. glabrata* (29, 41, 46, 48, 51).

The mechanisms causing *C. krusei* to be innately resistant to FLC are poorly understood. It has been shown that *C. krusei* Erg11p is significantly less susceptible to FLC inhibition than most other fungal Erg11p proteins (11, 32, 49). Other studies have shown that efflux pumps are at least partially responsible for the high levels of *C. krusei* resistance (4, 16, 25, 38, 50). Using degenerate primers designed against the highly con-

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TABLE 1. Fungal and yeast strains used in this study

Strain	Genotype	Source or reference ^a
<i>Candida</i> spp.		
<i>C. albicans</i> ATCC 10261	<i>C. albicans</i> isolate	ATCC
<i>C. krusei</i> B2399	<i>C. krusei</i> isolate	Centers for Disease Control
<i>C. krusei</i> IFO0011	<i>C. krusei</i> isolate	Institute for Fermentation (Osaka, Japan)
<i>C. krusei</i> 89.021	Isolate from human sputum	NZCDC
<i>C. krusei</i> 89.102	Isolate from porcine stomach	NZCDC
<i>C. krusei</i> 89.221	Isolate from a human cervical swab	NZCDC
<i>C. krusei</i> 90.147	Isolate from a human gastrectomy	NZCDC
<i>S. cerevisiae</i>		
AD1-8u ⁻	<i>MATα PDR1-3 ura3 his1 Δyor1::hisG Δsnq2::hisG</i> <i>Δpdr10::hisG Δpdr11::hisG Δycf1::hisG</i> <i>Δpdr3::hisG Δpdr5::hisG Δpdr15::hisG</i>	7
ADΔ	AD1-8u ⁻ , <i>Δura3</i>	20
ADΔ/ScERG11	ADΔ, <i>Δpdr5::pABC3-ScERG11</i>	This study
ADΔ/ScERG11-GFP	ADΔ, <i>Δpdr5::pABC3-ScERG11-GFP</i>	This study
ADΔ/CaERG11C	ADΔ, <i>Δpdr5::pABC3-CaERG11A</i>	20
ADΔ/CaERG11C-GFP	ADΔ, <i>Δpdr5::pABC3'-CaERG11A-GFP</i>	20
AD/CkERG11A	AD, <i>Δpdr5::pABC3-CkERG11A</i>	This study
AD/CkERG11A-GFP	AD, <i>Δpdr5::pABC3-CkERG11A-GFP</i>	This study
ADΔ/CkABC1g	ADΔ, <i>Δpdr5::pABC3-CkABC1g</i>	20
ADΔ/CkABC1g-GFP	ADΔ, <i>Δpdr5::pABC3-CkABC1g-GFP</i>	20
ADΔ/CkABC1	ADΔ, <i>Δpdr5::pABC3-CkABC1</i>	This study

^a ATCC, American Type Culture Collection; NZCDC, New Zealand Centre for Disease Control.

served C-terminal nucleotide binding domain 2 (NBD2) of pleiotropic drug resistance family (PDR) transporters such as *Saccharomyces cerevisiae* Pdr5p or *C. albicans* Cdr1p and Cdr2p, Katiyar and Edlind (16) successfully amplified and sequenced a 357-bp DNA fragment from *C. krusei* genomic DNA (gDNA). The gene was named *ABC1*. Northern analysis showed that *ABC1* expression was upregulated in the presence of azole antifungals, leading these authors to conclude that this potential efflux pump could be an important contributor to the innate FLC resistance of *C. krusei*, similar to the innate resistance mechanism found for *C. glabrata* (29). However, a recent report claims that no ABC transporters are involved in the innate FLC resistance of *C. krusei* (13).

In order to clarify uncertainties arising from these contradictory reports, we used inverse PCR to isolate and characterize the entire genes for both the azole drug target *ERG11* and the ABC transporter *ABC1* from wild-type *C. krusei* strains. We also analyzed their potential contributions to the innate azole resistance phenotype of *C. krusei* cells.

MATERIALS AND METHODS

Strains and culture conditions. The *C. krusei* isolates and yeast strains used in the present study are listed in Table 1. All *S. cerevisiae* strains created in the present study were based on strains AD1-8u⁻ (7) and ADΔ (20). All fungal strains were grown in yeast extract, peptone, and glucose (YPD) medium composed of 1% (wt/vol) Bacto yeast extract (Difco Laboratories, Detroit, MI), 2% (wt/vol) Bacto peptone (Difco), and 2% (wt/vol) glucose. Yeast transformants were selected on plates containing complete supplement mixture without uracil (CSM-ura; Bio 101, Vista, CA), 0.67% (wt/vol) yeast nitrogen base without amino acids (Difco), 2% (wt/vol) glucose, and 2% (wt/vol) agar (Difco). Plasmids were maintained in *Escherichia coli* strain DH5α. *E. coli* cells were grown in Luria-Bertani medium, to which ampicillin was added (100 µg/ml) as required.

Materials. Molecular biology reagents and restriction and modifying enzymes were from New England Biolabs (Beverly, MA) or from Roche Diagnostics N.Z., Ltd. (Auckland, New Zealand). High-pressure liquid chromatography-purified DNA oligonucleotides were purchased from Hermann Gbr Synthetische Bio-

moleküle (Denzlingen, Germany). PCR and DNA fragments were purified by using kits from Qiagen Pty., Ltd. (Clifton Hill, Victoria, Australia). gDNA was isolated from yeast by using a Y-DER yeast DNA extraction reagent kit (Pierce, Rockford, IL) or a Qiagen DNeasy tissue kit from Qiagen (Clifton Hill, Victoria, Australia). A random primer kit from Invitrogen New Zealand, Ltd. (Auckland, NZ), was used to radioactively label PCR fragments used in Southern and Northern experiments. Yeast were transformed by using the alkali-cation yeast transformation kit from Bio 101, taking into account the sensitivity of strain ADΔ to high concentrations of Li⁺ cations (20). All plasmids and DNA fragments were verified by DNA sequencing using a DYEnamic ET terminator cycle sequencing kit (v3.1; GE Healthcare UK, Ltd., Buckinghamshire, United Kingdom) and analyzed at the Micromon DNA sequencing facility (Monash University, Melbourne, Australia). PCRs used the high-fidelity KOD⁺ DNA polymerase (Toyobo, Osaka, Japan or Novagen, San Diego, CA).

Compounds. FLC (Diflucan; aqueous solution) was purchased from Pfizer Laboratories, Ltd. (Auckland, New Zealand). ITC and ketoconazole (KTC) were purchased from Janssen-Kyowa (Tokyo, Japan). Aureobasidin A was purchased from Takara Bio, Inc. (Shiga, Japan). Miconazole (MCZ), rhodamine 6G (R6G), rhodamine 123, nystatin, amphotericin B, cycloheximide, doxycycline, cerulenin, enniatin, oligomycin, flucytosine, terbinafine, nigericin, monensin, cytochalasin D, latrunculin A, and 4-nitroquinoline N-oxide were purchased from Sigma-Aldrich New Zealand, Ltd. (Auckland, New Zealand). FK506 was a gift from Astellas Pharma, Inc. (Tokyo, Japan), and the milbemycins α11, α20, α25, β9, and β11 were a gift from Sankyo Co., Ltd. (Tokyo, Japan).

Genetic manipulations. Plasmids used in the present study are listed in Table 2. Plasmid pBluescript II SK(+) (Stratagene, La Jolla, CA) was used as a cloning vehicle for all inverse PCR fragments. The *C. krusei* gDNA that was used as a template for inverse PCRs was prepared as follows: <1 µg of gDNA was digested to completion with restriction enzymes that gave bands of 1 to 5 kb in Southern blot experiments and then purified by phenol extraction, followed by ethanol precipitation, and redissolved in 100 µl of Milli-Q water. gDNA (<50 ng) was ligated (20-µl reaction) to form circular DNA molecules. Portions of the ligated gDNA (1 µl) were then used as templates for inverse PCRs. The primer pairs were designed to be close to, but pointing away from, each other (see P1 and P2 in Fig. 1A and C) and contained EcoRI restriction sites. The following PCR cycling protocol was used: 94°C for 5 min (94°C for 20 s, 55°C for 10 s, and 68°C for 1 min/kb) repeated 34 times, with a final extension time of 10 min at 68°C. The PCR amplicons consisted of the gDNA region (except for the small portion between the two primers) representing the restriction fragment that had been self-ligated. These PCR fragments were gel purified and then cut with the same restriction enzymes to split these fragments into their left (5') and right (3') arms,

TABLE 2. Plasmids used in this study

Plasmid	Description	Source or reference
pBluescript II SK(+)	Vector used for the cloning of all inverse PCR fragments	Stratagene
pABC3	pBluescript II SK(+)-based plasmid vector	20
pABC3-GFP	pABC3 containing a C-terminal GFP	20
pABC3-ScERG11	pABC3 containing the <i>ERG11</i> ORF from <i>S. cerevisiae</i> AD1-8u ⁻	This study
pABC3-ScERG11-GFP	pABC3-GFP containing the <i>ERG11</i> ORF from <i>S. cerevisiae</i> AD1-8u ⁻	This study
pABC3-CaERG11A	pABC3 containing the A allele of the <i>ERG11</i> ORF from <i>C. albicans</i> ATCC 10261	20
pABC3-CaERG11A-GFP	pABC3-GFP containing the A allele of the <i>ERG11</i> ORF from <i>C. albicans</i> ATCC 10261	This study
pABC3-CkERG11C	pABC3 containing the C allele of the <i>ERG11</i> ORF from <i>C. krusei</i> B2399	This study
pABC3-CkERG11C-GFP	pABC3-GFP containing the C allele of the <i>ERG11</i> ORF from <i>C. krusei</i> B2399	This study

respectively, and with EcoRI (the primers contained EcoRI sites). Left- and right-arm inverse PCR fragments were cloned separately into the multiple cloning site of pBluescript II SK(+) and sequenced.

Construction of yeast strains overexpressing heterologous membrane proteins. In order to study the function and localization of *C. krusei* Abc1p, the protein was overexpressed in *S. cerevisiae* ADAΔ. The construction of the *ABC1* overexpressing yeast strains ADAΔ/CkABC1g and ADAΔ/CkABC1g-GFP (both containing the gDNA open reading frame [ORF] including an 88-bp intron) or ADAΔ/CkABC1 (containing the cDNA ORF) has been described previously (20). Analogous to the heterologous overexpression of *C. krusei* Abc1p in *S. cerevisiae*

ADAΔ, the *ERG11* ORFs from *S. cerevisiae* AD1-8u⁻, *C. albicans* ATCC 10261, and *C. krusei* B2399 were cloned into plasmids pABC3 and pABC3-GFP as PacI/NotI fragments to create plasmids pABC3-ScERG11, pABC3-CaERG11A (20), and pABC3-CkERG11C as well as pABC3-ScERG11-GFP, pABC3-CaERG11A-GFP and pABC3-CkERG11C-GFP, respectively. All plasmids were cut with restriction enzyme AscI, and the resulting transformation cassettes were used to transform AD1-8u⁻ or ADAΔ. Ura⁺ transformants containing the cassette correctly integrated at the chromosomal *PDR5* locus were selected on CSM-ura plates and confirmed by colony PCR, using a previously described cloning procedure (20).

FIG. 1. Cloning and characterization of the *C. krusei* genomic loci containing *ERG11* and *ABC1*. Graphical representation of the genomic *ERG11* (A) and *ABC1* (C) loci of *C. krusei* strain B2399. Open arrows depict all potential ORFs identified. P1 and P2 are the primer pairs that were used in the inverse PCR experiments described in the text. Individual inverse PCR fragments that were cloned into vector pBluescript II SK(+) are numbered 1 to 4 underneath the restriction maps. The gray box within the *ABC1* ORF shown in panel C represents the region that was characterized previously by Katiyar and Edlind (16), while the black box indicates the experimentally verified intron in the *ABC1* ORF. Ba, BamHI; Bg, BglII; E, EcoRI; H, HindIII; P, PstI; S, SacI; X, XhoI. Restriction enzymes in italics in panel A (H and X) are enzymes that cut only once within the genomic region shown. (B) Autoradiograms for Southern blots performed on gDNA extracted from *C. krusei* strain B2399 that had been fully digested with the indicated restriction enzymes. The left-hand Southern blot was hybridized with the radioactively labeled fragment called the “5’ probe” shown as a light gray box on panel A, and the right-hand Southern blot was hybridized with the “3’ probe,” also shown as a light gray box on panel A. (D) Autoradiograms for Southern blots performed on gDNA extracted from *C. krusei* strains B2399 (left panel) and IFO0011 (right panel) using a radioactively labeled fragment of *ABC1* (light gray box in panel C named “probe”).

Purification and analysis of PM proteins. Plasma membrane (PM) fractions of *S. cerevisiae* cells were prepared as described previously (28). Protein samples (30 µg) were separated on 8% acrylamide gels and stained with Coomassie blue R250. *S. cerevisiae* strains expressing C-terminal green fluorescent protein (GFP) fusion proteins were examined by using a Zeiss 510 Axiovert 200 M inverted confocal laser scanning microscope (Otago Centre for Confocal Microscopy).

Functional analysis of wild-type and recombinant yeast strains. The susceptibility of yeast to antifungal agents was measured by a microdilution assay as described previously (14, 28). To be able to quantify the antifungal susceptibilities of *C. krusei* strains according to the Clinical and Laboratory Standards Institute (CLSI) standards and recommended breakpoints, we also determined the MICs for *C. krusei* by using the CLSI microdilution reference method (CLSI guidelines document M27-A2). Agarose diffusion assays were performed to test the susceptibility of *S. cerevisiae* strains overexpressing *C. krusei* Erg11p or Abc1p to different xenobiotics. In brief, 10 ml of YPD overnight culture of each test strain was diluted 1:20 into 3 ml of CSM medium, followed by growth at 30°C for another 4 h to mid-logarithmic growth phase (optical density at 600 nm [OD₆₀₀] of ~1, ~10⁷ cells/ml). The cells were diluted to an OD₆₀₀ of 0.008 in 5 ml of melted CSM medium containing 0.6% agarose (45°C) and overlaid on 20 ml of CSM agar-based medium. Whatman 3MM paper disks (5 mm in diameter) containing xenobiotics were placed onto the solidified top agarose medium, and the plates were incubated at 30°C for 48 h. The amounts of xenobiotics used are described in the figure legends.

Chemosensitization assays. The chemosensitization of a yeast strain overexpressing *C. krusei* Abc1p or *C. krusei* strains B2399 and IFO0011 to ITC was carried out as described previously (28). In brief, cells were cultured in CSM medium as for agarose diffusion assays. Each test strain was diluted to an OD₆₀₀ of 0.008 in 10 ml of melted CSM containing 0.6% agarose (45°C) with either no ITC (control to determine the toxicity of each drug) or ITC at 0.25× the MIC of ITC (MIC_{ITC}) determined on solid medium (see explanation below). The cell suspension was poured into a rectangular Omnitrax plate (126 by 86 by 19 mm; Nunc, Roskilde, Denmark) that contained 20 ml of CSM solidified with 0.6% agarose either without (control) or with 0.01 µg of ITC/ml. Whatman 3MM paper disks containing potential drug pump inhibitors (5 or 1 µg of milbemycin α1, α20, α25, β9, and β11, 1 µg or 0.2 µg of enniatin, 5 µg of FK506, or 25 nmol of oligomycin as indicated in the figure legends) were placed on the overlay, and the plates were incubated at 30°C for 48 h. The MIC_{ITC}s on solid media were between 20 to 100 times lower than the MIC_{ITC}s for the same strains determined in liquid media. This was determined by testing the growth of *S. cerevisiae* strains ADΔ and ADΔ/CkABC1g, as well as of *C. krusei* strains B2399 and IFO0011 on solid medium containing serial twofold dilutions of ITC. The MIC_{ITC} on solid medium was 0.0008 µg/ml for ADΔ compared to its MIC of 0.031 µg/ml in liquid medium, 0.04 µg/ml on solid medium compared to 4 µg/ml in liquid medium for ADΔ/CkABC1g, and 0.04 µg/ml compared to 0.5 or 1 µg/ml for *C. krusei* strains B2399 and IFO0011, respectively. This observation was specific for ITC only. We also tested the growth of the same strains on solid medium containing serial twofold dilutions of FLC or R6G. In both cases the MICs for all of the strains appeared to be similar on solid media to the MICs determined in liquid media.

Northern and Southern analysis. Total RNA was isolated from *C. krusei* B2399 cells by using the hot-phenol extraction method. Usually about 100 ODU (OD units; defined as the amount of cells corresponding to 1 ml of cells of an OD₆₀₀ of 1) of cells were harvested by centrifugation for 1 min at 3,000 × g, and the cells were washed once in ice-cold water and snap-frozen in liquid nitrogen and stored at -80°C. Samples (10 µg) of total RNA were separated on 1.2% denaturing agarose gels and stained with ethidium bromide (EtBr). The separated total RNA was photographed, immediately Northern blotted onto nylon⁺ membranes, and further processed according to standard protocols (40). For Southern blot analysis of *C. krusei* strains B2399 or IFO0011, 10 µg of gDNA was digested to completion with various restriction enzymes and separated on 0.8% agarose gels. Southern blotting of denatured gDNA, binding to nylon⁺ membranes, and hybridization of radioactive probes was carried out as described for the Northern blot analysis.

RESULTS

Isolation and sequence analysis of the *ERG11* ORF from two *C. krusei* strains. The entire *C. krusei* *ERG11* ORF was PCR amplified from gDNA templates of *C. krusei* strains B2399 and IFO0011 (Table 1). Sequencing the purified PCR fragments identified five and four single nucleotide polymorphisms (SNPs), respectively (Table 3). SNP C44T of strain IFO0011

TABLE 3. Sequences for individual *C. krusei* *ERG11* ORF alleles from strains B2399 and IFO0011^a

Strain and allele	SNP (nucleotide no.)					
	T/C (44)	T/C (642)	A/T (756)	T/C (939)	T/C (1389)	A/C (1470)
B2399						
A	C	C	T	T	T	C
B	C	C	A	T	C	A
C	C	T	A	C	C	C
IFO0011						
A	C	C	T	T	T	C
B	T	T	A	T	C	C

^a For simplicity, only the SNP nucleotides that are different between the sequences of individual *ERG11* ORF alleles are shown; the numbering of the nucleotides shown in parentheses starts with 1 for the A of the ATG start codon.

was the only nonsynonymous SNP leading to the conserved amino acid change A15V. Sequence alignment with a number of other fungal Erg11p proteins revealed that both amino acid variants exist in other fungal species (most *C. tropicalis* and *C. dubliniensis* Erg11p sequences have a valine, while most *C. albicans*, *C. glabrata*, and *S. cerevisiae* Erg11p sequences found in the GenBank database have an alanine; Fig. 2). Figure 2 also shows the location of secondary structure elements taken from the *Mycobacterium tuberculosis* MtCyp51p crystal structure, the structure that is often used for modeling eukaryotic Erg11p/Cyp51p proteins (9, 11, 36). While MtCyp51p is a soluble protein, eukaryotic Erg11ps and Cyp51ps contain an amino-terminal membrane anchor that targets them to the endoplasmic reticulum. PredictProtein, a bioinformatics program that predicts the protein topology of membrane proteins (39), predicted one amino-terminal transmembrane domain for *C. krusei* Erg11p located between amino acids S26 and A43 (Fig. 2). Thus, the first 25 amino acids are predicted to face the lumen of the endoplasmic reticulum, and the bulk of the protein (L44 to N528) faces the cytosol.

***C. krusei* B2399 contains three and IFO0011 contains two *ERG11* alleles.** In an attempt to identify the DNA sequences of individual *ERG11* alleles for strains B2399 and IFO0011, we cloned a number of individual *ERG11* ORFs into vector pABC3 and sequenced them. Unexpectedly, we identified three distinct alleles for *ERG11* of strain B2399 (Table 3) and two alleles for *ERG11* of strain IFO0011, as would be expected for a diploid strain (15, 43, 53, 54) (Table 3). Careful reexamination of the original sequencing chromatograms revealed a 2:1 ratio for all five B2399 SNPs and a 1:1 ratio for the four IFO0011 SNPs, as expected for strains containing three or two separate alleles, respectively. To be absolutely sure that the sequences for each individual allele were determined correctly and to distinguish clones representing individual alleles from clones that represented chimeras between different alleles, we sequenced 20 individual B2399 and 10 individual IFO0011 *ERG11* ORF clones from two separate PCRs using independently isolated gDNA as a template. This was necessary because we observed that a significant proportion (10 to 20%) of individually cloned PCR fragments turned out to be chimeric clones due to template switching between individual PCR cycles. The A allele ORFs of both strains were identical, while the other alleles (B and C alleles of strain B2399 and the B

FIG. 2. Multiple sequence alignment of *C. krusei* Erg11p with fungal Erg11ps, human Cyp51Ap and MtCyp51p. Using CLUSTAL W software *C. krusei* Erg11p was aligned with Erg11p from other important fungal pathogens such as *C. albicans* (CaErg11p), *C. dubliniensis* (CdErg11p), *C. tropicalis* (CtErg11p), *C. glabrata* (CgErg11p), *S. cerevisiae* (ScErg11p), *Aspergillus fumigatus* Erg11Ap (AfuErg11Ap) and Erg11Bp (AfuErg11Bp), and *C. neoformans* (CneErg11p) and compared to human Erg11p (HsCyp51Ap) and the soluble bacterial homolog *M. tuberculosis* Cyp51p (MtCyp51p), for which the crystal structure is known. Highly conserved residues are shown in red, conservative amino acid changes are shown in green, and nonconserved residues are shown in black. Shown above the alignments are all of the alpha helices (light gray boxes with their conventional letter designations) and beta strands (dark gray arrows with their conventional number designations) taken from the MtCyp51p structure (PDB ID number 2VKU). With the exception of MtCyp51p, all Erg11ps/Cyp51ps are predicted (using PredictProtein) to contain an amino-terminal membrane anchor domain spanning the endoplasmic reticulum membrane (TMD). The two gray boxes within the sequence alignments represent the two signature motifs common to all Erg11ps, with the most-conserved residues marked with an asterisk above the sequence. The four amino acid residues that are most often found to be associated with azole resistance in clinical *Candida* isolates are marked with round black dots above the individual amino acids. The plus sign above the essential cysteine residue marks the residue that forms the fifth heme iron ligand.

allele of strain IFO0011) were unique. The DNA sequences of all five *ERG11* alleles have been deposited with GenBank (accession numbers DQ903901 to DQ903905).

Characterization of 10 kb of gDNA surrounding the *C. krusei* *ERG11* locus. Assuming that *C. krusei* was diploid, we were surprised to identify three unique *ERG11* alleles in strain B2399. We initially thought that *C. krusei* may have acquired additional *ERG11* alleles via gene duplication events in the recent past. We hypothesized that the increased copy number for *ERG11*, together with its reduced affinity for azole antifungals, is the likely cause for its innate azole resistance phenotype. To test this hypothesis, we created a detailed map of the chromosomal *ERG11* locus to find evidence for repeats of *ERG11* in close proximity to each other. First, we performed Southern blot analysis of gDNA from B2399 using two radioactive probes (Fig. 1A and B). Strain IFO0011 gave identical results (data not shown). Using this information together with the *ERG11* ORF sequence obtained we were able to perform two rounds of inverse PCR (using primer pairs P1 and P2) to amplify, clone, and sequence fragments 1, 2, and 4 shown in Fig. 1A (see also Materials and Methods), thus yielding nearly 10 kb of DNA sequence surrounding, and including, the *ERG11* ORF.

Four ORFs were identified as illustrated in Fig. 1A. ORFs I (399 bp; 132 amino acids) and II (471 bp; 156 amino acids) were predicted to encode mitochondrial ribosomal proteins MrpL38p and Mrp49p, respectively. ORF IV showed highest homology to the rRNA processing protein 45 (Rrp45p; 1,233 bp; 410 amino acids) that is part of the 3'-5' exonuclease complex (exosome complex). ORFs I, II, and III are all transcribed in the same direction, while ORF IV is encoded by the opposite strand. No evidence for any other ORFs or extra copies of *ERG11* was found. An alternative explanation for the presence of three different *ERG11* alleles in strain B2399 is that this strain contains three *ERG11*-containing chromosomes.

***C. krusei* B2399 is aneuploid with at least three *ERG11*-containing chromosomes.** If strain B2399 has three separate *ERG11*-containing chromosomes rather than multiple copies of *ERG11* on the same chromosome, we should be able to identify three distinct alleles for the whole 10-kb chromosomal region identified above. This was indeed the case. In order to exclude PCR artifacts, more than 10 individual clones from independent PCR amplifications for each of the DNA fragments 1, 2, 3, and 4 shown in Fig. 1A were sequenced, and all contained allele-specific SNPs, including their three overlapping regions. Therefore, we could identify the individual DNA sequences for all three alleles across the whole 10 kb. All B2399 SNPs were verified by directly sequencing the PCR fragments amplified from gDNA, and again all exhibited the expected 2:1 ratio as described above. Aligning the three alleles revealed 74 unique SNPs (18 SNPs specific for the A allele, 31 SNPs specific for the B allele, and 25 SNPs specific for the C allele). On average, there was one allele-specific SNP every 400 bp, which is similar to the SNP frequencies we found for different *C. albicans* strains (the average allele-specific SNP frequency for *CDR1* from six different *C. albicans* strains was one SNP/425 bp and one SNP/160 bp for *CDR2* [14]). Closer inspection revealed that more than 4 kb of the 3' end of the gDNA sequences (starting just downstream of ORF IV as

illustrated in Fig. 1A) of both the A and the C alleles of strain B2399 were identical, while the B allele contained 21 of its 31 unique SNPs in that region. As expected, the SNP frequency was lower within the four ORFs (only 12 of the 74 unique SNPs lay within the four identified ORFs), leading to an average SNP frequency within ORFs I to IV of an individual allele of one SNP/900 bp. The sequences for all three B2399 alleles have been deposited with GenBank (accession numbers EU296425 to EU296427).

Isolation and characterization of the genomic locus of *ABC1* of strains B2399 and IFO0011. Using degenerate primers against the highly conserved C-terminal Walker A and B motifs of fungal ABC transporters, Katiyar and Edlind (16) were able to isolate two small (357-bp) DNA fragments representing part of the C-terminal NBD2 of two *C. krusei* ABC transporters, which they named *ABC1* and *ABC2*. *ABC1* showed highest homology to the well-characterized multidrug efflux transporters *S. cerevisiae* Pdr5p and *C. albicans* Cdr1p and Cdr2p (16). These authors also showed that the expression of *ABC1*, unlike *ABC2*, was strongly induced by azole antifungals. This led to the conclusion that Abc1p is likely associated with the innate FLC resistance phenotype of *C. krusei*. We were able to confirm that *C. krusei* *ABC1* encodes a multidrug efflux pump by isolating the whole gene and heterologously overexpressing Abc1p in *S. cerevisiae*, leading to an ~100-fold-decreased susceptibility to azole antifungals (20). The present study, however, is the first detailed description of the isolation and characterization of *ABC1*. Again, due to a lack of *C. krusei* sequence information, we used inverse PCR to isolate and characterize the entire genomic *ABC1* locus. Two rounds of inverse PCR were needed to isolate and sequence the whole *ABC1* gene of strain B2399 (see Materials and Methods and Fig. 1C). Cloning and sequencing fragments 1, 2, 3, and 4 yielded the sequence for the entire *ABC1* gene, including 664 bp upstream of the ATG start codon and 983 bp downstream of the TAA stop codon (Fig. 1C). Sequence analysis of the *ABC1* ORF predicted an 88-bp intron that was experimentally verified (20). Using these sequencing data the *ABC1* ORF of strain IFO0011 was also amplified and sequenced. The sequences for the *ABC1* ORF of strains B2399 and IFO0011 were identical except for one synonymous SNP (T4549C).

Surprisingly, no SNPs were detected across the whole *ABC1* ORF, including the promoter and terminator regions of strains B2399 (6,301 bp) and IFO0011 (5,255 bp), suggesting that both strains are homozygously diploid in *ABC1* or, alternatively, both strains are monosomic for *ABC1*. Southern blot analysis of gDNA from *C. krusei* strains B2399 and IFO0011 revealed a restriction fragment length polymorphism for EcoRI showing two bands (6 and 8 kb) for EcoRI-digested gDNA (lanes E in Fig. 1D), even though the restriction map of *ABC1* (Fig. 1C) predicted only one band larger than 5.5 kb. These data suggest that, unlike for *ERG11*, both B2399 and IFO0011 strains are homozygous diploid for *ABC1*. The gDNA and cDNA sequences for *ABC1* of *C. krusei* strain B2399 and the gDNA sequence for *ABC1* of strain IFO0011 have been deposited with GenBank (accession numbers DQ903906, DQ903907, and EU296340). Despite extensive efforts, it was not possible to amplify *ABC2* by inverse PCR.

***C. krusei* *ABC1* is highly homologous to *C. albicans* and *C. glabrata* *CDR1* and *S. cerevisiae* *PDR5*.** Performance of a "pro-

A				B			
NBD1				Predicted Membrane Topology			
	WalkerA	ABC signature	WalkerB				
	*						
ABC1	GRPGAGCS	IRGVSGGERKRVIAE	QCWDN	ABC1	533	–	550
CDR1	GRPGAGCS	VRGVSGGERKRVIAE	QCWDN	CDR1	516	–	533
CDR2	GRPGAGCS	VRGVSGGERKRVIAE	QCWDN	CgCDR1	513	–	530
CgCDR1	GRPGSGCT	VRGVSGGERKRVIAE	QCWDN	PDR5	524	–	541
PDR5	GRPGSGCT	VRGVSGGERKRVIAE	QCWDN				

FIG. 3. *C. krusei* Abc1p is an ABC transporter highly homologous to other well-characterized fungal multidrug efflux pumps. (A) Alignment of the highly conserved Walker A, Walker B, and ABC signature motif of NBD1 and NBD2 of Abc1p from *C. krusei* B2399 with the NBD1 and NBD2 motif of the multidrug efflux pumps *C. albicans* Cdr1p, Cdr2p, *C. glabrata* CgCdr1p, and *S. cerevisiae* Pdr5p. The conserved residues identical to the Abc1p sequence are shaded in gray. The asterisks mark the highly conserved cysteine and lysine residues of the NBD1 and NBD2 domains as described in the text. (B) List of the predicted transmembrane segments TMS1 to TMS12 for the PDR family proteins Abc1p, Cdr1p, CgCdr1p, and Pdr5p using the PredictProtein software program available from Columbia University.

tein-blast" search with the predicted Abc1p protein sequence (1,521 amino acids) showed that Abc1p shared highest homology with *C. albicans* Cdr1p (59% identical and 75% homologous), Cdr4p (59 and 75), Cdr2p (57 and 74), and Cdr3p (55 and 72), followed by *C. glabrata* CgCdr1p (54 and 71) and *S. cerevisiae* Pdr5p (53 and 69), all belonging to the cluster I type ABC transporter family with the predicted topology of two amino-terminal NBDs, each followed by a transmembrane domain (TMD) containing six individual transmembrane spanning segments (TMSs) in the order: NBD-TMD-NBD-TMD. PredictProtein revealed that not only is Abc1p highly homologous to other fungal multidrug efflux pumps but the predicted locations of its individual transmembrane segments (TMS 1 to 12) also aligned well (plus or minus one amino acid) with the predicted locations of individual TMSs for Cdr1p, CgCdr1p, and Pdr5p (Fig. 3B). The NBDs were also highly conserved (including Walker A, ABC signature, and Walker B motifs; Fig. 3A). Abc1p NBD1 showed only one conserved amino acid change at the amino-terminal end of the ABC signature motif compared to the other efflux pumps (I317V) with two additional amino acid changes found in the Walker A motifs for both CgCdr1p and Pdr5p (A210S and S213T; Fig. 3A). The NBD2 domains of *C. krusei* Abc1p and Cdr1p, Cdr2p, CgCdr1p, and ScPdr5p only had one conserved amino acid change (L1027 of Abc1p, Cdr1p, and Cdr2p was replaced by a V in CgCdr1p and Pdr5p) (Fig. 3A). It seems that *C. krusei* Abc1p shares the same asymmetry in its two NBDs that is a unique feature of the fungal PDR family of ABC transporters (10, 52). It too contains a cysteine (C212) instead of the usually essential lysine in the Walker A motif of its NBD1 and an essential lysine (L906) in the Walker A motif of NBD2 (Fig.

3A), suggesting the functional nonequivalence of the two NBDs of Abc1p as described for CgCdr1p and ScPdr5p (10, 52). Finally, comparing Abc1p with the small part of Abc1p previously isolated (16) revealed three conservative amino acid differences (I990M, D1018E, and V1019L). These changes are likely due to natural strain variations.

Functional characterization of Abc1p and Erg11p of *C. krusei* strain B2399 overexpressed in *S. cerevisiae* ADA. The overexpression of Abc1p in *S. cerevisiae* ADA, a host strain that is highly susceptible to many xenobiotics, resulted in a 100-fold increase in resistance to azole antifungals (20). To confirm that Abc1p is a multidrug efflux transporter, we tested whether its overexpression in AD/CkABC1g could confer increased resistance to 20 other xenobiotics, some of which are known to be substrates of other fungal efflux pumps (Fig. 4). As expected, overexpression of Abc1p conferred increased levels of resistance to a range of unrelated xenobiotics. Apparent substrates of Abc1p included, aside from all of the azoles tested, cerulenin, cycloheximide, nigericin, monensin, rhodamine 123, R6G, cytochalasin D, and latrunculin A. In contrast, aureobasidin A, 4-nitroquinoline N-oxide, doxycycline, nystatin, amphotericin B, flucytosine, and terbinafine appeared not to be substrates of Abc1p (Fig. 4).

The resistance pattern for the AD strain overexpressing *C. krusei* Erg11p was also determined (Fig. 4). *C. krusei* Erg11p was functionally expressed in the AD strain, and this resulted in increased levels of resistance to azole antifungals only, as expected for the overexpression of the azole drug target. It appeared, however, that the overexpression of *C. krusei* Erg11p led to a substantially lower increase in resistance to ITC (Fig. 4, lower panel; there is a much smaller reduction of the growth-

FIG. 4. Expression of *C. krusei ABC1* or *ERG11* confers multidrug resistance on *S. cerevisiae*. Cells (ADA, top row; ADA/CkABC1g, central row; or AD/CkERG11C, bottom row) were grown to mid-logarithmic growth phase and plated on CSM agar plates (pH 7.0). 3MM Whatman filter disks that contained different xenobiotics were then placed onto the agar surface, and the plates were incubated at 30°C for 48 h. The following amounts of xenobiotics were applied to the disks: FLC, 7 µg (23 nmol); ITC, 250 ng (0.36 nmol); KTC, 150 ng (0.28 nmol); MCZ, 12.5 ng (0.026 nmol); nystatin (NYT), 2.5 µg (22 nmol); amphotericin B (AMB), 80 µg (87 nmol); flucytosine (5FC), 2.5 µg (19 nmol); terbinafine (TRB), 15 µg (46 nmol); cerulenin (CER), 502 ng (2 nmol); cycloheximide (CHX), 105 ng (0.37 nmol); nigericin (NIG), 10 µg (8 nmol); monensin (MON), 8 µg (12 nmol); rhodamine 123 (R123), 50 µg (131 nmol); R6G, 7.5 µg (16 nmol); cytochalasin D (CYT-D), 10.2 µg (20 nmol); latrunculin A (LAT-A), 100 ng (0.24 nmol); aureobasidin A (AUR-A), 8 µg (7 nmol); 4-nitroquinoline N-oxide (4-NIT), 0.8 µg (4 nmol); trifluoperazine (TRI), 300 µg (620 nmol); and doxycycline (DOX), 1,200 µg (2.5 µmol).

inhibitory zone around the ITC-containing disk compared to all other azoles tested). Liquid MIC results confirmed that AD/CkERG11C cells were only twice as resistant to ITC as the control AD strain compared to 8 to 16 times more resistant than the AD strain to FLC, VRC, and MCZ (Table 4).

PM preparations of *S. cerevisiae* cells overexpressing Abc1p showed high levels of expression of the 170-kDa Abc1p protein (Fig. 5A). Although expression of Abc1p was achieved for both the gDNA *ABC1* ORF (Fig. 5A, lane 2; *ABC1* gene contains an 88-bp intron), as well as the cDNA *ABC1* ORF (Fig. 5A, lane 3), higher Abc1p expression was achieved (relative to the predominant 100-kDa PM protein Pma1p) from cells containing the *ABC1* cDNA ORF. For comparison, Erg11p proteins (~60 kDa) from three different fungal species (*S. cerevisiae* ADA, *C. albicans* ATCC 10261, and *C. krusei* B2399) were also expressed in *S. cerevisiae* AD or ADA strains (Fig. 5B, lanes 2, 3,

and 4, respectively). It was evident that *C. krusei* B2399 Erg11p was expressed at significantly lower levels than Erg11p from *S. cerevisiae* or *C. albicans*, although all of them appeared to be expressed functionally, as shown in Fig. 4 and Table 4.

***ERG11* and *ABC1* are constitutively expressed in *C. krusei*.** To study mRNA expression patterns for *ABC1* and *ERG11*, total RNA from B2399 cells grown under a number of different conditions was isolated and subjected to Northern blot analysis (Fig. 6). The housekeeping gene *ACT1* from *C. krusei* B2399 was used as an internal control. Equal loading of total RNA was also confirmed by EtBr staining of the gels. Both *ABC1* and *ERG11* were constitutively expressed in logarithmically growing cells, and their expression was dramatically reduced in cells about to enter the diauxic growth phase (Fig. 6A, lanes 8, 3, 12, and 13 [numbers representing the actual OD₆₀₀ values]). Exposing early-logarithmic-phase cells (OD₆₀₀ = 1) for 60 min to different azole antifungals had no significant effects on *ABC1* mRNA levels (Fig. 6B). In contrast, *ERG11* mRNA levels increased severalfold within 1 h in response to concentrations 2× the MIC of ITC, MCZ, and VRC and 1× the MIC of FLC (Fig. 6B; higher concentrations of FLC [> 200 µg/ml] could not be achieved). During a 320-min time course of exposure of logarithmically grown cells (OD₆₀₀ = 1) to FLC at a concentration of 1× MIC, no induction in the expression of *ABC1* mRNA was observed, whereas *ERG11* expression increased significantly compared to control cells (Fig. 6C). The highest levels of *ERG11* expression were reached after 80 min of exposure to FLC. This peak of expression coincided with the time when cell growth started to slow down compared to the untreated control cells (data not shown). Decreased levels of *ABC1* expression for the control cells at the later time points (160 and 320 min, left panel of Fig. 6C) coincided with the entry of cells into the diauxic phase. To test how *ABC1* and *ERG11* expression responded to defined stress conditions, exponentially grown cells (OD₆₀₀ = 1) were harvested, resuspended in fresh media, and exposed to the following for a further 20 min: fresh medium only (control), 2 mM H₂O₂, pH 7.0, 500 mM NaCl, or 42°C. *ERG11* expression was dramatically repressed under all of the conditions tested, but the stron-

TABLE 4. Susceptibility of *C. krusei* isolates and *S. cerevisiae* strains overexpressing different fungal *ERG11* alleles or the multidrug efflux pump *ABC1* from *C. krusei* to azoles measured in CSM (pH 7.0) by liquid microdilution assay

Strain	MIC (µg/ml) ^a				
	FLC	ITC	KTC	MCZ	VRC
<i>C. krusei</i>					
B2399	256 (32)	0.5 (0.25)	2 (0.5)	2 (4)	1 (0.5)
IFO0011	256 (128)	1 (0.25)	2 (1)	4 (4)	1 (1)
89.021	256 (32)	1 (0.25)	4 (0.5)	4 (2)	2 (0.5)
89.102	256 (32)	2 (0.25)	4 (0.5)	4 (2)	2 (0.5)
89.221	256 (32)	1 (0.125)	2 (0.5)	4 (2)	1 (0.5)
90.147	256 (128)	1 (0.25)	4 (1)	4 (4)	2 (1)
<i>S. cerevisiae</i>					
ADA	0.5	0.031	0.031	0.0078	0.0078
ADA/ScERG11	2.0 (4)	0.25 (8)	0.063 (2)	0.031 (4)	0.063 (8)
ADA/CaERG11A	2.0 (4)	0.125 (4)	0.125 (4)	0.063 (8)	0.031 (4)
AD/CkERG11C	4 (8)	0.063 (2)	0.063 (2)	0.125 (16)	0.063 (8)
ADA/CkABC1g	64 (128)	4 (128)	2 (64)	0.5 (64)	0.5 (64)

^a The numbers in parentheses for the *C. krusei* strains indicate the MICs for these strains as determined by the CLSI method, while the numbers in parentheses for the *S. cerevisiae* strains indicate the fold increases in the MICs for recombinant *S. cerevisiae* strains versus the control strain ADA.

FIG. 5. Expression of Abc1p and Erg11p proteins in *C. krusei* and recombinant *S. cerevisiae*. (A and B) Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) gels of membrane fractions (30 μ g; containing PMs together with endoplasmic reticulum) that were isolated from *S. cerevisiae* AD Δ cells overexpressing Abc1p (A) or different fungal Erg11p proteins (B). All strains were grown in YPD medium and harvested in late logarithmic growth phase. Membrane proteins in panel A were isolated from the control strain AD Δ (lane 1) and from strains AD Δ /CkABC1g (genomic clone containing 1 intron, lane 2) and AD Δ /CkABC1 (cDNA clone, lane 3). (B) SDS-PAGE analysis of membrane fractions (30 μ g) obtained from AD Δ control strain (lane 1), AD Δ /ScERG11 (lane 2), AD Δ /CaERG11 (lane 3), and AD/CkERG11C (lane 4). (C and D) SDS-PAGE analysis of protein fractions (30 μ g) that were isolated from *C. krusei* B2399 cells grown in YPD medium without FLC (C) or with FLC at a concentration of $1\times$ MIC_{FLC} (D) and then harvested at 0, 1, 2, and 3 h. Gray rectangular boxes highlight the 170-kDa protein bands for Abc1p in panels A, C, and D, as well as the 60-kDa protein bands for Erg11p in panels B, C, and D.

gest repression was observed in cells exposed to oxidative stress and high temperature (Fig. 6D). NaCl (500 mM) or a change in pH did not repress *ABC1* expression. In contrast, oxidative stress and a high temperature repressed the expression of *ABC1* significantly, as it did for *ERG11* (Fig. 6D). Serum cholesterol has been shown to reduce azole susceptibilities of fungi such as *C. glabrata* (27) or *A. fumigatus* (56) by complementing the cells' need for ergosterol through the uptake of cholesterol from the serum. Therefore, we also tested whether ergosterol at concentrations similar to those found in serum would have any effect on *ERG11* or *ABC1* expression. Incubating *C. krusei* cells for 60 min in the presence of ergosterol had no detectable

effect, however, on the expression of *ERG11* or *ABC1* (Fig. 6E). In conclusion, the expression patterns for *ERG11* were as expected. Challenging *C. krusei* cells with azole antifungals at concentrations equal to or higher than their MIC led to a dramatic increase in the expression of *ERG11*, but only after more than 1 h, when presumably the cell membranes started to become depleted of ergosterol. In contrast, the downregulation of *ERG11* in response to stress was much more rapid (20 min). While *ABC1* expression was constitutive and relatively high (easy to detect) in growing cells, it was not affected by azole antifungals and the response of *ABC1* expression to stress was also more specific than that of *ERG11*. While

FIG. 6. Expression of *ERG11* and *ABC1* mRNA in *C. krusei* B2399 grown under a variety of growth conditions. Total RNA was isolated from *C. krusei* B2399 cells that were grown under a number of different growth conditions. The radioactively labeled probes for *ERG11* and *ABC1* are shown in Fig. 1A and C, respectively, and the probe that was used for *ACT1* is the same as described previously (16). The results for *ABC1* are shown in the top panel, and the same RNA samples were used for *ERG11* shown directly underneath them. *ACT1* mRNA levels were used as an internal control (third panel) and EtBr-stained ribosomal bands shown in the bottom panel indicate RNA loading. (A) Growth-phase-dependent expression of *ERG11* and *ABC1* mRNA of B2399 cells grown in YPD. The OD₆₀₀ values at the times of harvest are shown above the corresponding lanes. (B and E) Expression of *ABC1* and *ERG11* mRNA in logarithmically grown *C. krusei* B2399 cells exposed for 60 min to different azole antifungals (C, untreated control; FLC, $1\times$ the MIC_{FLC}; VRC, $2\times$ the MIC_{VRC}; MCZ, $2\times$ the MIC_{MCZ}; and ITC, $2\times$ the MIC_{ITC}) (B) or ergosterol (–erg, sterol solvent Tween 80 control; +erg, 10 μ g of ergosterol/ml) (E). (C) Expression of *C. krusei* *ABC1* and *ERG11* mRNA in the absence (–FLC) or presence (+FLC) of FLC at a concentration of $1\times$ the MIC_{FLC} for the indicated times. (D) Expression of *ABC1* and *ERG11* mRNA in logarithmically grown *C. krusei* B2399 cells exposed for 20 min to different stress conditions: C, no stress control; H₂O₂, 2 mM H₂O₂; NaCl, 500 mM NaCl; pH 7.0, CSM_{pH 7.0}; and 42°C.

***Candida krusei* *ERG11* promoter**

-276
 GAAAAAAAAAAAAAAAAAACGCATTTTTTTTTTCTCGAATTTTATAACGACTATATGTGGATTCTCGTATAACTTTGACGAGCCGATAATAC
 TCGTTGCGACAAACGATGTTTTTTTTTCTTCTTGGTGTGTTGTTCAATTAATGTGTGTTTTTCATAACTTTTCATAAATATATAAGAACGCGCAATTTT
 ACCGTTTTCTTCTACATTTGATCCCATTCATCCAAAGGAGGTTTCTGAACTGTATTCTCCTCTAGCAACAACATG

INV 15 bp SRE

***Candida albicans* *ERG11* promoter**

-297
 GAATTTGATAAAGAGAAAAAGAAATATTGGGTTTGTCTTGTATTCATATCGTACCGATTATGTCGTATATCTTTTTTCAATGTCAATTTGAGAAGC
 AGAACGAAACAAAAATAAAAAATAATATCATACAATAATTTTTCTTCATCTTACTTCTTTCTTTCAATCTTTAATAAATCAATTTTTATATATAAAT
 AGACAAAGAAAGGAATTCATCGTTATTCTTTCCATATTACTTGTCTCTTTTATTATATATATAAGTTTCTTTCAAGAAGATCATAACTCAATATG

INV 13 bp SRE

***Candida glabrata* *ERG11* promoter**

-236
 AACAACTGCAATTTTGAAAAAAAAAATGAAAAAAAAATTTATCATCGTATATTCACCTCGAAGAACCGTATACTCATCTCGTATAATCAGTAGTC
 AAGACTTGTGCTAAACATCTTTACAAAAAATGTATATAATGGGCGATCCCTTCATGTCCATTGTCTTGAAATTTCTTGTCTCATTTGCTATTTTCGATAC
 TACAAACCACATAAATCAAAAAAACTACAATAACATG

SRE 29 bp SRE

FIG. 7. The *C. krusei* *ERG11* promoter contains a conserved ARE. The first 200 to 300 bp of DNA sequence upstream of the ATG start codon of *C. krusei* *ERG11* is compared to the *ERG11* promoter sequences of *C. albicans* and *C. glabrata*. The ATG start codons are underlined and the predicted TATA boxes are shown in bold face. SREs or their inverted sequences (INV) are shown overlined (SRE) or underlined (parts of SRE and their matching inverted INV sequences), respectively. The INVs and SREs of *C. albicans* *ERG11* have been experimentally verified and contain the ARE that is bound by the zinc cluster transcription factor Upc2p leading to the activation of transcription. The SRE and INV elements for *C. glabrata* and *C. krusei* shown for comparison await experimental verification.

ERG11 was repressed under all stress conditions tested, *ABC1* was only repressed in response to oxidative stress and high temperature.

Identification of conserved promoter elements for *ERG11*. The data presented above show that the expression pattern for *C. krusei* *Erg11p* is very similar to that observed for other fungal azole drug targets such as *S. cerevisiae* and *C. albicans* *Erg11p* (31, 44). Recently, Oliver et al. (31) identified an azole responsive element (ARE) that is responsible for the azole-dependent upregulation of *C. albicans* *ERG11* (Fig. 7). Part of the ARE element is the sterol responsive element (SRE; TCG-TATA) that is found in a number of *ERG* genes in *S. cerevisiae* and *C. albicans* (23, 31). The zinc cluster transcription factor Upc2p has been shown to bind to the SRE element in the *C. albicans* *ERG11* promoter, leading to the azole-induced expression of *Erg11p* (31). The ARE for *C. albicans* *ERG11* consists of the SRE and an almost perfect inverted repeat (INV) of that element 13 bp upstream (Fig. 7). Both of these elements are important for the azole-induced Upc2p-dependent upregulation of *ERG11* in *C. albicans* (31). We discovered a very similar ARE-like element that was conserved in all *ERG11* alleles at nearly the identical position (−204 versus −236 upstream of the ATG start codon) in the *ERG11* promoter of *C. krusei*. The spacing between the INV and the SRE elements was slightly larger (15 bp versus 13 bp, Fig. 7). Unlike the *ERG11* promoters for *C. albicans* and *C. krusei*, the search for an ARE in the *C. glabrata* *ERG11* promoter revealed that it possesses two direct repeats of the SRE separated by 29 bp (bp −153 and −189). Clearly, the SRE is conserved between *S. cerevisiae*, *C. albicans*, *C. glabrata*, and *C. krusei*. The only

difference appears to be the way that two of these elements are positioned relative to each other.

Since *C. krusei* *ABC1* is highly homologous to *S. cerevisiae* *PDR5*, *C. glabrata* *CDR1* and *PDH1*, and *C. albicans* *CDR1* and *CDR2*, its promoter might be expected to possess pleiotropic drug resistance elements (PDREs) found in the *S. cerevisiae* *PDR5* and *C. glabrata* *CDR1* and *PDH1* promoters (17, 46, 48) or drug-responsive elements found in *C. albicans* *CDR1* and *CDR2* (8). However, no PDREs were present in the *ABC1* promoter, and this might explain why *ABC1* expression is not induced by FLC (for a review of the family of fungal specific zinc cluster transcription factors such as Pdr1p and Pdr3p of *S. cerevisiae* and CgPdr1p and Tac1p of *C. albicans* recognizing PDREs and drug-responsive elements, see reference 24).

Expression of *Erg11p* and *Abc1p* proteins in *C. krusei* B2399 cells. To confirm that not only *ERG11* mRNA but actual *Erg11p* expression is induced by azole antifungals, membrane fractions were isolated from cells that were grown over a 3-h period at the same FLC concentration and under the same conditions that were used for the Northern blot experiments. Figure 5C and D show membrane fractions isolated from B2399 cells grown in the absence (Fig. 5C) or presence (Fig. 5D) of FLC. As was found for *ERG11* mRNA, a band of the expected size for the *Erg11p* protein (~60 kDa) was strongly induced by FLC (lower boxed areas in Fig. 5C and D). However, while *ERG11* mRNA levels were highest 80 min after exposure to FLC, *Erg11p* protein levels were highest 3 h after induction. In contrast, only a faint band at the expected size for the *Abc1p* protein (170 kDa) could be detected. The amounts of the 170-kDa protein band were very similar between FLC

FIG. 8. Confocal microscopy of AD or ADA cells overexpressing *C. krusei* Abc1p or fungal Erg11p proteins. Multidrug efflux pump *C. krusei* Abc1p localized to the PM, whereas the fungal azole drug target proteins Erg11p from *S. cerevisiae*, *C. albicans* and *C. krusei* localized exclusively to internal structures mainly around the nucleus reminiscent of rough endoplasmic reticulum. Light microscopy images of the cells are shown above their confocal images, and fused images of the light microscopy images with the confocal images are shown underneath the confocal images. The two *CaERG11* images have been taken at different sensitivities in order to see more detail in the right hand image. The right-hand *CaERG11* image was taken at the same setting as the *CkERG11* image.

treated and untreated cells and also appeared to be lowest at the 3-h time point when the cells started entering the diauxic phase (upper boxed areas in Fig. 5C and D). This expression pattern appeared to be consistent with the Northern results but was somewhat different compared to *C. glabrata* cells which, in response to FLC, clearly overexpress both a 61-kDa protein, Erg11p, and a 170-kDa band corresponding to Cdr1p (29). The amounts of *C. krusei* Erg11p expressed in *S. cerevisiae* AD/CkERG11C were comparable to the highest levels of Erg11p expressed in *C. krusei* cells grown in the presence of FLC (compare lane 4 of Fig. 5B with lane 3 of Fig. 5D).

Erg11p is localized in the endoplasmic reticulum and Abc1p in the PM. To test the localization of the fungal Erg11p proteins and the *C. krusei* multidrug transporter Abc1p expressed in *S. cerevisiae* strains AD or ADA, C-terminal GFP fusions of these proteins were created, and their localization was analyzed by confocal microscopy. Addition of the C-terminal GFP tags to Erg11p and Abc1p did not affect the function of either protein (data not shown for Erg11p; see reference 20 for Abc1p). All three fungal Erg11p proteins were properly localized in internal structures reminiscent of membranes of the rough endoplasmic reticulum surrounding the nucleus (Fig. 8). Judging from the intensity of the fluorescent signals (different exposure times were used for fluorescent images in Fig. 8), Erg11ps from both *S. cerevisiae* and *C. albicans* were expressed at significantly higher levels than Erg11p from *C. krusei*. This correlates well with the amounts of the untagged versions of these proteins expressed in AD and ADA strains (Fig. 5B). Confocal microscopy also revealed that the C-terminal Abc1p-GFP fusion expressed in ADA cells is localized in the PM, as expected.

Functional characterization of *S. cerevisiae* cells overexpressing *C. krusei* Erg11p and Abc1p. Disk diffusion assays

indicated that Abc1p is a multidrug efflux pump that can transport azole antifungals (Fig. 4). To get a better understanding of the individual contributions of *C. krusei* Abc1p and Erg11p to the overall FLC resistance phenotype of wild-type *C. krusei* cells the azole susceptibilities of *S. cerevisiae* cells overexpressing a selection of fungal Erg11p proteins or the multidrug transporter Abc1p to five azole antifungals (FLC, ITC, KTC, MCZ, and VRC) was quantified by measuring liquid MICs (Table 4). Assuming that Erg11p protein expression levels for the same construct are more or less constant in each MIC assay (a constitutive overexpression system is being used), comparing the different azole MICs of each fungal Erg11p-overexpressing strain can be used as a direct measure of its relative affinities for different azole antifungals. For example, high MICs (relative to the control strains AD or ADA) indicate a low affinity of an azole to its drug target Erg11p, whereas low MICs indicate a relatively higher affinity of the azole to that particular fungal Erg11p. The average fold increase for ADA/ScERG11 was fourfold (twofold for KTC, fourfold for MCZ and FLC, and eightfold for ITC and VRC). Erg11p from *C. albicans* was overexpressed at levels very similar to those for Erg11p from *S. cerevisiae* (Fig. 5B), and it led to very similar levels of increased resistance (~4-fold) for all five azoles tested (Table 4). This indicates that Erg11ps from *S. cerevisiae* and *C. albicans* have very similar affinities to the five azoles tested. Although Erg11p from *C. krusei* was expressed at much lower levels than Erg11p from *S. cerevisiae* or *C. albicans* (compare lanes 2, 3, and 4 of Fig. 5B), it conferred the highest levels of resistance to FLC and MCZ and similar levels of resistance to VRC and KTC (Table 4). This suggests that *C. krusei* Erg11p has lower affinities for FLC and MCZ than Erg11p from *S. cerevisiae* or *C. albicans*.

The overexpression of the efflux pump Abc1p in *S. cerevisiae*

ADA led to between 64- and 128-fold-increased levels of resistance for all azoles tested (Table 4). Abc1p from *C. krusei* appears to be a very efficient azole transporter.

For comparison, the azole susceptibilities of six *C. krusei* isolates are also shown in Table 4. Since host *S. cerevisiae* AD cannot be grown in the medium recommended by CLSI for the determination of azole MICs in yeast, though we wanted to compare the azole susceptibilities of these *C. krusei* isolates with the azole susceptibilities of all *S. cerevisiae* strains shown in Table 4, and because we wanted to determine the MICs for all strains grown in the medium that was used for the expression studies shown in Fig. 5 and 6, we used CSM (pH 7.0) medium for all measurements. However, in order to interpret the azole susceptibilities of the *C. krusei* strains according to the interpretative breakpoints defined for the CLSI protocol, we also determined the azole susceptibilities of all six *C. krusei* strains according to the recommended CLSI method. These data are shown in parentheses in Table 4. According to the CLSI protocol, all six *C. krusei* isolates were classified as either resistant ($n = 2$) or susceptible dose dependent (SDD; $n = 4$) to FLC and KTC; all isolates except *C. krusei* 89221 (susceptible) were SDD to ITC, while all six strains were sensitive to VRC. No breakpoints are available for MCZ.

Abc1p inhibitors chemosensitize *C. krusei* and Abc1p overexpressing *S. cerevisiae* strains to FLC and ITC. We have previously described a number of specific small molecule inhibitors of fungal multidrug transporters, including *C. krusei* Abc1p (20). To test our hypothesis that Abc1p contributes significantly to the innate azole resistance of *C. krusei*, we tested the effect of these inhibitors on the azole susceptibilities of *C. krusei* strains B2399 and IFO0011. We used CSM agar plates containing sub-MIC concentrations of ITC (25% of MIC_{ITC}) and measured the growth of cells around filter disks that contained known, or suspected, pump inhibitors placed onto the surface of agar plates seeded with *C. krusei* or AD/CkABC1g cells. All eight small molecule inhibitors tested inhibited Abc1p pump activity and chemosensitized *S. cerevisiae* cells overexpressing Abc1p to ITC (Fig. 9A). The strongest inhibition was obtained with milbemycins α 11, α 20, and α 25, milbemycin β 11, and FK506. *C. krusei* strains B2399 and IFO0011 were also chemosensitized by the same inhibitors with the exception of milbemycin α 11 which showed only minor chemosensitization. Milbemycin β 9, enniatin, and oligomycin showed little chemosensitization of cells overexpressing Abc1p to ITC and had no detectable effect on the *C. krusei* strains. The chemosensitization of cells overexpressing Abc1p and *C. krusei* strain B2399 by milbemycin α 20 and β 11 was quantified by using a checkerboard susceptibility assay (26). Milbemycin α 20 fully inhibited the pump function of Abc1p at concentrations greater than 2 μ g/ml and fully chemosensitized *S. cerevisiae* ADA/CkABC1g cells to FLC and ITC so that their susceptibilities became identical to ADA cells (Fig. 9Bc and d). The same concentrations of milbemycin α 20 also showed synergistic effects with FLC or ITC on *C. krusei* strain B2399. Milbemycin α 20 (2.5 μ g/ml) reduced the MIC_{FLC} for strain B2399 to 64 μ g/ml and the MIC_{ITC} to 0.125 μ g/ml (Fig. 9Ba and b). The chemosensitization experiments were also performed with milbemycin β 11, leading to very similar results (data not shown). The fractional inhibitory concentration indices (FICI) (26) for the combination of milbemycin α 20 and

either FLC or ITC for ADA/CkABC1g cells were 0.112 or 0.104, respectively, and the FICI values for the same combinations for *C. krusei* B2399 cells were 0.35 or 0.298, respectively. FICI values are a quantitative measure for combination therapies and values of <0.5 indicate synergy between two drugs (26, 30, 45).

DISCUSSION

All *C. krusei* strains used in this study were isolated from different sources and prior to the clinical use of FLC in the early nineties. Strain B2399 was obtained as a reference *C. krusei* strain from the CDC. Strain IFO0011 was isolated in Japan and the other strains used here are New Zealand isolates from different parts of the human body or from pig intestine. Despite the diversity of the environmental sources, all six strains exhibited very similar azole susceptibilities. They were all fully resistant or SDD to FLC and KTC and SDD to ITC, with the exception of strain 89221, which was sensitive to ITC. However, all strains appeared susceptible to VRC, a broad-spectrum triazole antifungal. Although *C. krusei* Erg11p has a significantly diminished sensitivity for FLC, ITC, and VRC and possibly other azole antifungals (11, 32), conflicting reports exist on whether other factors such as ABC multidrug efflux transporters or the reduced uptake of azoles also contribute to the innate FLC resistance of *C. krusei* (25, 49, 50). Although some studies have described large differences in the steady-state levels for different azoles in *C. krusei* (25, 50), a recent report claimed that no ABC transporter could be involved in the innate azole resistance phenotype (13).

In the present study we have shown that the functional overexpression of *C. krusei* Erg11p in our hypersusceptible host *S. cerevisiae* AD (deleted in seven ABC transporters) at levels similar to the highest, azole-induced, expression levels reached in *C. krusei* still resulted in a FLC-susceptible yeast (MIC_{FLC} = 4 μ g/ml compared to MIC_{FLC} = 256 μ g/ml for wild-type *C. krusei* using the same CSM [pH 7.0] medium). This finding led us to the hypothesis that *C. krusei* Erg11p alone is not sufficient to cause the innate azole resistance of most *C. krusei* strains. Functional overexpression of *C. krusei* Abc1p in *S. cerevisiae* ADA led to a 64- to 128-fold-increased level of resistance to FLC (MIC_{FLC} = 64 μ g/ml) and other azoles. The fact that Abc1p is constitutively expressed in *C. krusei*, that it is highly homologous to known multidrug efflux pumps of related yeast, and that it confers high levels of resistance to azole antifungals when overexpressed in *S. cerevisiae* points to Abc1p being a significant contributor to the low azole susceptibility of *C. krusei*. To test that hypothesis, we attempted to create an *ABC1* knockout strain of *C. krusei*. Due to the lack of *C. krusei* auxotrophic markers, we set out to adapt the *SAT1* flipper/dominant selection marker technique, which has been used to knock out genes in clinical *C. albicans* isolates and in *C. glabrata* (37), to inactivate *ABC1* in *C. krusei*. Unfortunately, all attempts to create *ABC1* knockout strains failed due to the high level of *C. krusei* resistance to nourseothricin (even at 500 μ g/ml). So, instead, we used Abc1p inhibitors to test whether Abc1p contributes significantly to the FLC resistance of *C. krusei* cells. This was achieved by demonstrating that *C. krusei* cells became four to eight times more sensitive to FLC and ITC when they were grown in the presence of

FIG. 9. Abc1p-specific inhibitors chemosensitize *C. krusei* cells and *S. cerevisiae* cells overexpressing Abc1p to ITC and FLC. A *S. cerevisiae* ADΔ cells overexpressing Abc1p, or *C. krusei* cells, were analyzed by agarose diffusion assays using CSM solidified with 0.6% agarose and containing either no ITC (top row of agar plates; control to test the toxicity of inhibitors) or ITC at 0.25× the agar MIC_{ITC} for each test strain (0.01 μg/ml). Whatman 3MM paper disks containing the indicated amounts of drug pump inhibitors (1 μg each of milbemycins α11, α20, α25, β9, and β11; 5 μg of FK506; 0.2 μg of enniatin; and 25 nmol of oligomycin) were placed onto the plate for ADΔ/CkABC1g cells. Five times more of the milbemycin inhibitors (5 μg) and enniatin (1 μg) were used for *C. krusei* strains B2399 and IFO0011. The plates were incubated at 30°C for 48 h. (B) The MIC_{FLC} and MIC_{ITC} of *C. krusei* B2399 cells (a and b) and ADΔ/CkABC1g cells (c and d) were measured at various concentrations of the Abc1p inhibitor milbemycin α20 as described in Materials and Methods.

specific nontoxic inhibitors of Abc1p. There may be other pumps contributing to the azole resistance of *C. krusei*, and it is possible that the fourfold increased susceptibilities of *C. krusei* to FLC and ITC conferred by milbemycins α20 and β11 is caused through the inhibition of another related pump and not Abc1p. However, a number of observations indicate that this is unlikely to be the case. As shown in Fig. 9A and as previously shown for FLC (20), the same inhibitors that best chemosensitized *S. cerevisiae* cells overexpressing *C. krusei* Abc1p to ITC (milbemycins α20, α25, and β11 and FK506) also best chemosensitized *C. krusei* to ITC. This is significant because when we tested the same set of compounds against a range of other homologous fungal efflux pumps (Pdr5p, Cdr1p

and Cdr2p, CgCdr1p, and CgPdh1p) each pump was inhibited by a unique subset of these compounds (20). Even Cdr1p and Cdr2p, highly homologous efflux pumps from *C. albicans*, were inhibited by a very distinct set of xenobiotics. In addition, the concentrations of milbemycins α20 and β11 (<2 μg/ml) required to fully chemosensitize *C. krusei* to FLC and ITC were similar to those required to fully inhibit *C. krusei* Abc1p expressed in *S. cerevisiae*. Taken together, it appears that *C. krusei* Abc1p contributes significantly to azole resistance of *C. krusei*.

Sequence alignments and functional studies suggest that *C. krusei* Abc1p is an ortholog of *S. cerevisiae* Pdr5p. Interestingly, in our hands Abc1p expression was not stimulated by the

presence of different azoles, as has been reported previously for Cdrp1, CgCdr1p (46), and also for Abc1p (15). One reason why *ABC1* mRNA induction was seen previously by Katiyar and Edlind (16) may be because these authors used more than 10 times the concentrations of azoles, concentrations that were up to 100 times their MICs.

Despite extensive efforts, we were not able to isolate *ABC2*, the second *C. krusei* ABC transporter reported by Katiyar and Edlind (16). The reason for this is not clear and requires further investigation.

In view of the data provided here and elsewhere (11, 32, 49), it seems that the lower susceptibility of *C. krusei* Erg11p really is a major reason for the innate azole resistance of *C. krusei*. In order to investigate what makes it so much less susceptible to azoles than most other fungal Erg11ps, we compared *C. krusei* Erg11p with other fungal Erg11ps, human Cyp51Ap, and MtCyp51p (a CLUSTAL W alignment of these proteins is shown in Fig. 2). The most obvious difference between fungal Erg11ps and MtCyp51p, as well as human Cyp51Ap, is a 15- to 24-amino-acid insertion between the K^{'''} and L helices compared to MtCyp51p. Significantly, the insertion is greatest for CkErg11p (an extra 5 amino acids; Fig. 2). The most recent three-dimensional structure for MtCyp51p can be found in PDB (2VKU) (9). It is interesting that this insertion is very close to two important amino acid residues (G464 and R467 of the CaErg11p sequence highlighted with black dots above the alignment in Fig. 2) that, when mutated to S464 and/or K467, lead to azole resistance in clinical *C. albicans* isolates (36, 42). Only a further three amino acids downstream is the essential cysteine (C470) that forms the fifth ligand of the iron in the prosthetic heme group. This insertion could be important for the lower azole susceptibility of *C. krusei* Erg11p compared to all other fungal Erg11ps. Since this region is predicted to be close to the base of the heme, it is possible that a change in this region somehow affects the environment adjacent to the heme and potentially the binding of azoles to the heme. Because this region is absent in MtCyp51p upon which fungal Erg11p structures are modeled, it is not possible to visualize the changes. Whether this region is important for the reduced azole susceptibility of *C. krusei* Erg11p can only be answered by solving the fungal Erg11p structure.

Another interesting and somewhat surprising discovery was that *C. krusei* strain B2399 contained three *ERG11* alleles. However, no obvious correlation between the *ERG11* copy number and FLC resistance was observed. The trisomy in the *ERG11*-containing chromosome observed for *C. krusei* strain B2399 is clearly due to the presence of three separate alleles that did not arise from a gene duplication event but possibly through the mating of two diploid *C. krusei* strains, followed by an imperfect loss of chromosomes resulting in aneuploidy. This is reminiscent of *C. albicans* and its imperfect sexual reproduction that can also lead to aneuploidy. *Cryptococcus neoformans* is another important fungal pathogen that has been shown to possess somewhat similar properties. The mating of *C. neoformans* serotype A *MATa* with serotype D *MATα* variant strains was recently shown to produce diploid and sometimes aneuploid AD hybrid strains with increased fitness to UV (21, 22). In contrast, a recent study of the effects of aneuploidy in *S. cerevisiae* showed that the possession of one extra copy of any of its 16 chromosomes leads to a range of phenotypes such as

defects in cell cycle progression, protein synthesis, protein folding, and biomass production (47). An extra copy of any of the chromosomes tested induced a typical stress response and in this case aneuploidy led to reduced fitness. It may be that the aneuploidy of *C. krusei* B2399, like that of *C. neoformans* variants, confers phenotypic characteristics that lead to some fitness advantage. The presence of three chromosomes in one of our isolates raises the possibility that *C. krusei* is able to reproduce sexually; however, we are not aware of any reports that show that *C. krusei* isolates can be mated under laboratory conditions. Recently, Jacobsen et al. (15) used multilocus sequence typing to determine the population structure of a large collection of *C. krusei* isolates, and they, too, found evidence for a departure from the mainly clonal reproduction pattern that is seen for *C. albicans* strains. These authors concluded that investigating the ability of *C. krusei* to undergo sexual reproduction was warranted.

To summarize, our data support the model that the innate FLC resistance phenotype of *C. krusei* is mainly a combination of two factors: the low sensitivity of the drug target Erg11p to azole antifungals, which causes a rather high basal level of resistance to azole antifungals, and the constitutive expression of the multidrug efflux pump Abc1p that most likely tips the balance in favor of innate azole resistance for most *C. krusei* strains. One could therefore speculate that the future use of broad-spectrum triazoles such as VRC that are effective against *C. krusei* but are also substrates of Abc1p could lead to the rise of VRC resistant *C. krusei* strains that overexpress Abc1p analogous to the resistance mechanisms described for other *Candida* species. In addition, the discovery that one of our six *C. krusei* isolates possessed three copies of the *ERG11* gene (and we have evidence that one other strain contains three copies of *ERG11* as well) leads us to speculate that a number of *C. krusei* strains are aneuploid.

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