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MECHANISMS OF RESISTANCE

Genomewide Elucidation of Drug Resistance Mechanisms for Systemically Used Antifungal Drugs Amphotericin B, Caspofungin, and Voriconazole in the Budding Yeast

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ABSTRACT There are only a few antifungal drugs used systemically in treatment, and invasive fungal infections that are resistant to these drugs are an emerging problem in health care. In this study, we performed a high-copy-number genomic DNA (gDNA) library screening to find and characterize genes that reduce susceptibility to amphotericin B, caspofungin, and voriconazole in *Saccharomyces cerevisiae*. We identified the *PDR16* and *PMP3* genes for amphotericin B, *the RMD9* and *SWH1* genes for caspofungin, and the *MRS3* and *TRI1* genes for voriconazole. The deletion mutants for *PDR16* and *PMP3* were drug susceptible, but the other mutants had no apparent susceptibility. Quantitative-PCR analyses suggested that the corresponding drugs upregulated expression of the *PDR16*, *PMP3*, *SWH1*, and *MRS3* genes. To further characterize these genes, we also profiled the global expression patterns of the cells after treatment with the antifungals and determined the genes and paths that were up- or downregulated. We also cloned *Candida albicans* homologs of the *PDR16*, *PMP3*, *MRS3*, and *TRI1* genes and expressed them in *S. cerevisiae*. Heterologous expression of *Candida* homologs also provided reduced drug susceptibility to the budding yeast cells. Our analyses suggest the involvement of new genes in antifungal drug resistance.

KEYWORDS amphotericin B, antifungal agents, caspofungin, drug resistance, genomics, multidrug resistance, voriconazole

Invasive fungal infections are mostly seen in patients who have been exposed to cancer chemotherapeutics or immune suppressors (1–4). There are many antifungal drugs used in local skin infections; however, the number of systemically used drugs is limited due to their side effects. Recent reports show that infectious fungi may develop resistance to new systemically used antifungals, which poses a higher risk for patients. Thus, we intended to search for drug resistance genes for amphotericin B (AmB), caspofungin (CSP), and voriconazole (VOR) by a genomic-DNA (gDNA) screening method.

Amphotericin B (5), a polyene macrolide antibiotic, was first discovered as a natural product of *Streptomyces nodosus* (6) and disrupts membrane permeability by binding to membrane lipids and forming pore-like structures (6). Binding of AmB to ergosterol is proposed as the main mechanism of AmB action, and the leakage of ions through the pores is considered a separate but complementary effect (7).

As an alternative to AmB, a new antifungal drug, CSP, is also used against pathogenic yeasts and molds, such as *Candida* spp. and *Aspergillus* spp. CSP is a member of the echinocandins that inhibits the synthesis of (1,3)-β-D-glucan in the cell wall (8).

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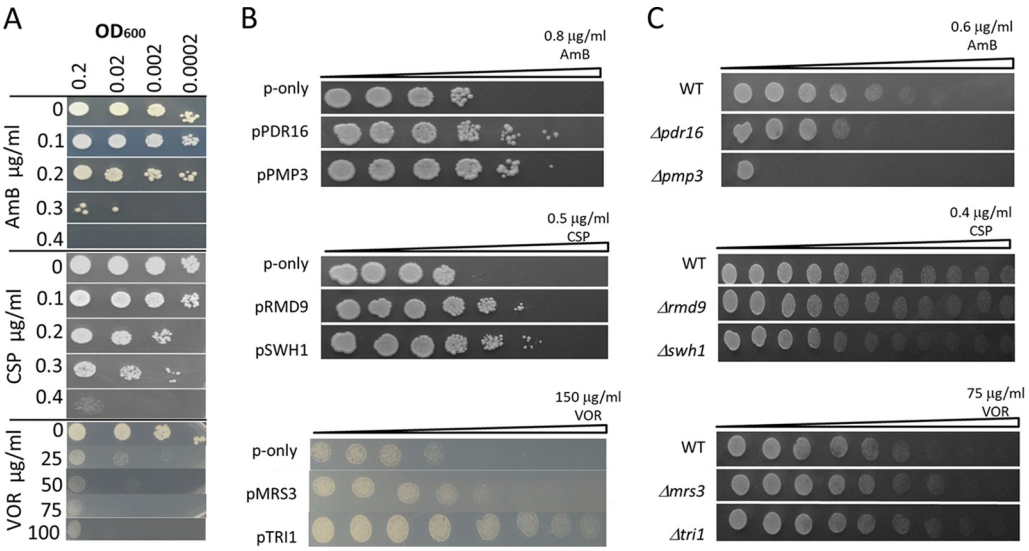

FIG 1 Spotting assay for AmB, CSP, and VOR resistance genes. (A) Spotting assay of wild-type cells growing on agar plates with the indicated concentrations of antifungal drugs. The cells were serially diluted, and a 5-μl aliquot was dropped for each spot. (B) Wild-type cells overexpressing *PDR16* and *PMP3*, *RMD9* and *SWH1*, or *MRS3* and *TRI1* were grown on plates containing a gradient of AmB, CSP, and VOR, respectively. Wild-type cells with an empty plasmid were used as controls (p-only). (C) Spotting assay for deletion mutants. The plates were incubated at 30°C for 3 days after inoculation and photographed.

Noncompetitive inhibition of the (1,3)-β-D-glucan synthase (Fks1) enzyme by CSP both reduces fungal cell growth as a fungistatic effect and causes the instability to osmotic pressure that kills the fungal cells (9).

Another new antifungal is the triazole VOR, which was produced as a fluconazole derivative (10). VOR directly targets the iron atom and inhibits the cytochrome P450-dependent lanosterol 14-α-demethylase (Erg11) enzyme (11). Lack of ergosterol production disrupts fungal growth.

Identification of genes that play a role in antifungal drug susceptibility may provide further prognostic information, which may lead to improvement in the development of new agents or increase the efficacy of antifungals. In this study, we identified six genes that provide reduced susceptibility to antifungal drugs when overexpressed, and most of these genes have not been associated with any drug resistance mechanisms previously. We also cloned *Candida albicans* homologs of these genes, expressed them in budding yeast, and showed that they were involved in reduced drug susceptibility.

RESULTS

Screening of gDNA library and identification of drug resistance genes. We first determined the lethal doses for AmB, CSP, and VOR under our conditions on yeast nitrogen base-2% glucose (YNB) agar medium for the *Saccharomyces cerevisiae* yeast strain BY4741 (12). We plated a series of serially diluted cell solutions onto agar plates containing different concentrations of each drug. The cells were not able to tolerate more than 0.4 μg/ml of AmB or 0.4 μg/ml of CSP. However, we noticed that the yeast strain that we used (BY4741) showed residual growth on high doses (100 μg/ml) of VOR (Fig. 1A, bottom). Next, we determined the MIC_{100} value (complete inhibition of growth) for AmB and the MIC_{50} values for CSP and VOR analyses in liquid YNB medium following the EUCAST microdilution procedure. AmB had a MIC_{100} value of 0.4 μg/ml. CSP and VOR had similar MIC_{50} values, 0.10 μg/ml and 0.15 μg/ml, respectively. In general, our MIC analyses and spotting assays were in agreement with each other. For screening purposes, we transferred gDNA-transformed cells onto YNB plates containing the lethal doses of the drugs. We observed four AmB colonies, two CSP colonies, and three VOR colonies showing reduced susceptibility to the corresponding drugs. Plasmids were recovered from all of these colonies and used to transform fresh wild-type

TABLE 1 Genomic sequences responsible for AmB, CSP, and VOR resistance

Drug name	Colony no.	Chromosome coordinates	Covered genes[a]
AmB	A1, A2, A4	Chromosome IV, bp 1009734–1019446	DON1, YDR274C, BSC2, **PMP3**, MTH1, ARS430, YDR278C, part of RNH202
	A3	Chromosome XIV, bp 210826–218065	BNI4, CSL4, **PDR16**, part of ELA1
CSP	C1	Chromosome I, bp 190872–196459	YAR035C-A, **SWH1**
	C2	Chromosome VII, bp 303197–308230	**RMD9**, YGL108C, YGL109W, MLC1, ARC1
VOR	V1	Chromosome X, bp 164101–159624	YJL133C-A, **MRS3**, YJL132W
	V2, V3	Chromosome XIII, bp 737351–741548	**TRI1**, RNH1

[a]Resistance genes are shown in boldface.

(WT) cells to confirm that they were responsible for the growth in the presence of the drugs. After confirmation, plasmids were sequenced from both sites of the expression cassettes, and the genes that they harbored were identified by BLAST search using the *Saccharomyces* Genome Database. Genes that were present in each expression cassette are listed in Table 1.

Three of the AmB-resistant colonies contained the same expression cassette, which covered a 9.7-kb DNA region on chromosome (Chr) IV involving the *PMP3* gene. The other resistant colony contained a 7.2-kb DNA region on Chr XIV covering the *PDR16* gene. Since *PDR16* is a member of the multidrug resistance family and the *PMP3* gene was previously associated with AmB resistance (13, 14), we directly cloned these genes onto separate vectors to confirm that they were responsible for the reduced susceptibility that we observed. The overexpression of the *PMP3* and *PDR16* genes in WT cells reduced AmB susceptibility (Fig. 1B).

We also cloned candidate genes for CSP and VOR from the library cassettes and overexpressed them in WT cells to identify which ones were responsible for the drug tolerance. Overexpression of the *RMD9* and *SWH1* genes reduced susceptibility to CSP, and overexpression of the *MRS3* or *TRI1* gene reduced susceptibility to VOR (Fig. 1B).

Rmd9 plays a role in mitochondrial mRNA processing (15) and has not been associated with any drug resistance mechanisms. Swh1 is a homolog of human oxysterol binding protein and was found to be important in resistance to haloperidol, a psychiatric drug (16).

One of the genes that reduced voriconazole susceptibility, *MRS3*, is a member of the mitochondrial carrier family and mediates iron transport across the inner membrane (17). The other gene, *TRI1*, encodes a SUMOylated protein with unknown function (18). Both VOR genes have not been implicated in any drug resistance previously.

We reasoned that if the overexpression of these genes reduces drug susceptibility, then their deletion should render cells susceptible to the drugs. Therefore, we analyzed the deletion mutants for the ability to grow in the presence of the drugs and observed that absence of *PDR16* and *PMP3* sensitized cells to AmB, but the deletion mutants for neither CSP- nor VOR-related genes were susceptible to the drugs (Fig. 1C).

Transcriptional analyses of genes. Our genetic-screening analyses suggested that the *PMP3*, *PDR16*, *RMD9*, *SWH1*, *MRS3*, and *TRI1* genes reduced drug susceptibility when overexpressed from a high-copy-number plasmid. Our next question was whether these genes were transcriptionally responsive to drug treatment. Gene-based transcriptional analyses were performed using a real-time quantitative-PCR (qPCR) approach. Cells were treated with a sublethal dose of the drugs (0.2 μg/ml AmB, 0.01 μg/ml CSP, or 75 μg/ml VOR) for 2 h during the exponential growth phase. Control groups were not exposed to any drugs. Expression levels of both the *PDR16* and *PMP3* genes were found to be significantly upregulated by AmB (2.5-fold and 15-fold, respectively) (Fig. 2A). The voriconazole tolerance gene, *MRS3*, also had significant (17-fold) upregulation; however, the *TRI1* gene was not activated by the drug (Fig. 2B). Treatment with CSP upregulated *SWH1* expression by 1.5-fold yet downregulated *RMD9* expression by 0.4-fold (Fig. 2C).

Additionally, whole-genome expression profiles of cells were monitored by microarray analyses to ascertain the cellular processes that were affected by the drugs. Genes

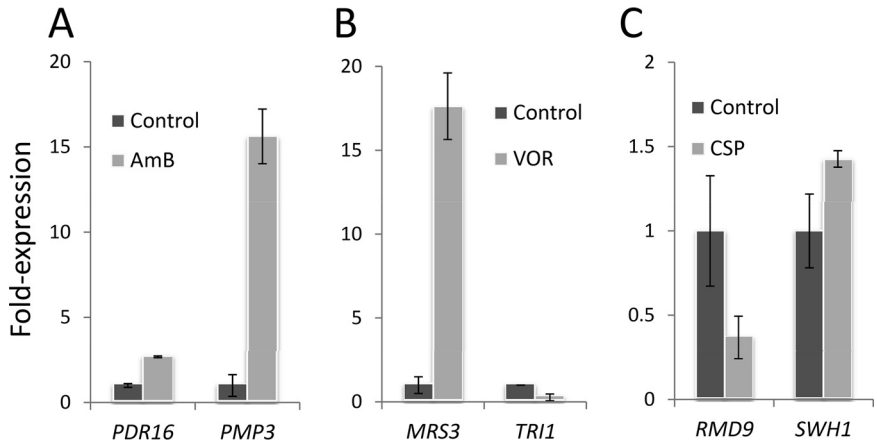

FIG 2 Real-time qPCR analyses for resistance genes. Wild-type cells were incubated with a sublethal dose of AmB (0.2 μg/ml) (A), VOR (75 μg/ml) (B), and CSP (0.01 μg/ml) (C) for 2 h and analyzed for their transcript levels. The *ACT1* gene used as an internal control. The significance of the differences was calculated by Student's *t* test ($P < 0.05$). Each gene was analyzed in two biological samples with triplicate readings. The error bars indicate SD.

that were upregulated or downregulated more than 2-fold were analyzed by MIPS (Munich Information Center for Protein Sequences) functional classification. The primary response of yeast cells to AmB was to activate carbohydrate and energy metabolism (see Table S1 in the supplemental material). In contrast, the amino acid metabolism and ion transport genes were inhibited (see Table S2 in the supplemental material). Similarly, CSP activated the carbohydrate metabolism genes and inhibited DNA synthesis and replication genes (see Tables S3 and S4 in the supplemental material). On the other hand, VOR increased the levels of meiosis genes rather than energy metabolism and also inhibited lipid metabolism (see Tables S5 and S6 in the supplemental material).

Membrane-related physiological and morphological analyses. The plasma membrane forms the first line of defense against external stressors, and its lipid composition plays essential roles in its integrity and functionality. Both the Pmp3 and Pdr16 proteins are associated with the cell membrane and have roles in lipid dynamics. Additionally, antifungal drugs mainly target the plasma membrane in pathogenic fungi. Therefore, we wanted to determine membrane-related functions, such as membrane potential, cytosolic pH, and cytotoxic-cation tolerance, in the mutants and the wild-type cells overexpressing these genes.

First, we monitored the membrane potentials of the cells using a fluorescence assay based on the membrane potential-dependent distribution of the dye diS-C_3(3) (3,3′-dipropylthiacarbocyanine iodide). As shown in Fig. 3A, most of the mutants had membrane potentials similar to that of the wild-type cells, except the Δ*rmd9* mutants, which were hyperpolarized. When the *RMD9* gene was overexpressed, the membrane potential was returned to the normal value (Fig. 3C). Overexpression of the *PDR16*, *PMP3*, *MRS3*, and *TRI1* genes led to normal membrane potentials (Fig. 3B and D). However, *SWH1* overexpression hyperpolarized the cells (Fig. 3C).

Next, we exposed cells to NaCl, LiCl, and the cationic drugs hygromycin B (HygB), tetramethyl ammonium (TMA), and spermine. As shown in Fig. S1A in the supplemental material, Δ*pmp3* cells were salt, HygB, and TMA susceptible, but Δ*pdr16* cells showed no susceptibility to any of the conditions tested. The hypersensitivity of Δ*pmp3* mutants to salt, HygB, and TMA was in close agreement with previously published data (19). The other deletion mutants were not susceptible to the cationic agents. Similarly, overexpression of these genes in wild-type cells caused no observable phenotypes on plates containing cations and drugs, except that *SWH1* overexpression caused TMA resistance (see Fig. S1B and C).

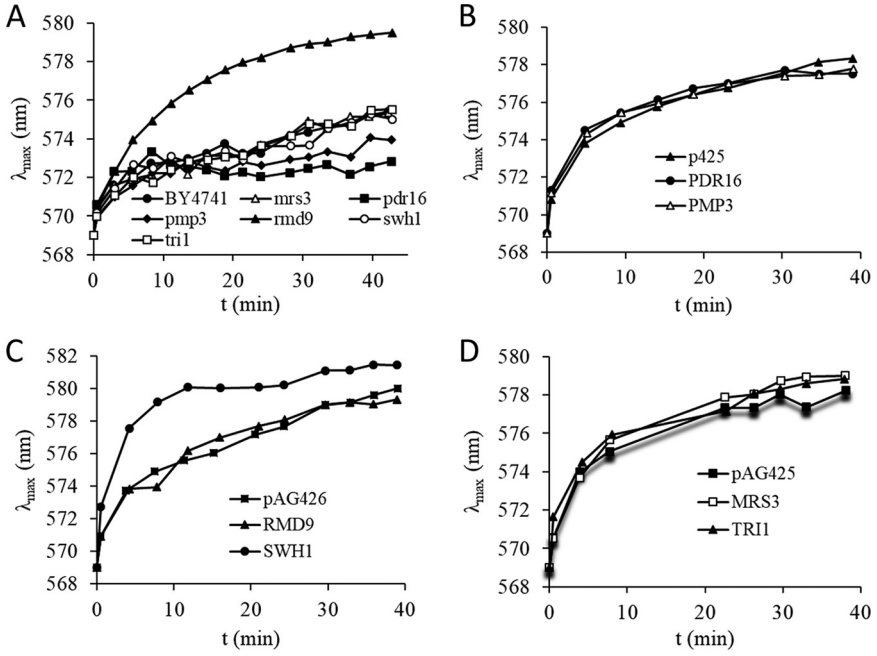

FIG 3 Plasma membrane potential measurements. (A) Relative membrane potentials of the deletion mutants. BY4741 wild-type cells were used as the control. (B) Membrane potentials of wild-type cells overexpressing *PDR16* and *PMP3* genes. Cells carrying empty plasmids (p426) were used as the control. (C) Membrane potentials of wild-type cells overexpressing *RMD9* and *SWH1* genes. Cells carrying empty plasmids (pAG425) were used as the control. (D) Membrane potentials of wild-type cells overexpressing *MRS3* and *TRI1* genes. Cells carrying empty plasmids (pAG425) were used as the control.

As the next step, we tested whether the drug tolerance of the mutants was correlated with changes in intracellular pH. When we measured the intracellular pH with pHluorin expression (20), we did observe that the Δ*pmp3*, Δ*pdr16*, and Δ*rmd9* mutants were more acidic than wild-type cells (Fig. 4).

Expression of *Candida* orthologous genes in budding yeast reduces susceptibility to the drugs. To test if the susceptibility genes had similar roles in pathogenic *C. albicans*, we amplified the open reading frames of *C. albicans* orthologs by PCR and expressed them in wild-type *S. cerevisiae* cells. There were no *C. albicans* orthologs for the CSP resistance genes *RMD9* and *SWH1*, and thus, we did not include these genes in analyses. Expression of both *C. albicans PMP3* (*CaPMP3*) and *CaPDR16* reduced susceptibility to AmB treatment (Fig. 5A). The *CaPMP3* gene was previously shown to reduce AmB susceptibility in *S. cerevisiae* (13, 14). However, the *CaPDR16* gene was not associated with reduced AmB susceptibility previously. This study showed that the *CaPDR16* gene was also crucial to reduce AmB susceptibility in the heterologous host

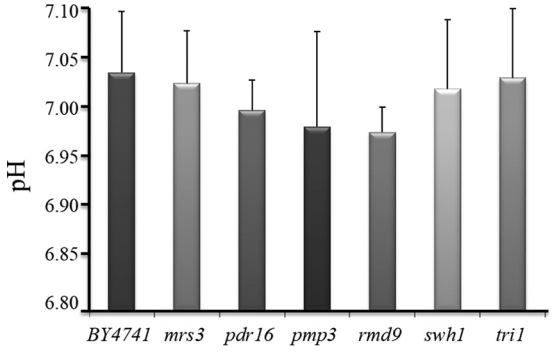

FIG 4 Intracellular pH values of the mutants. The error bars indicate SD.

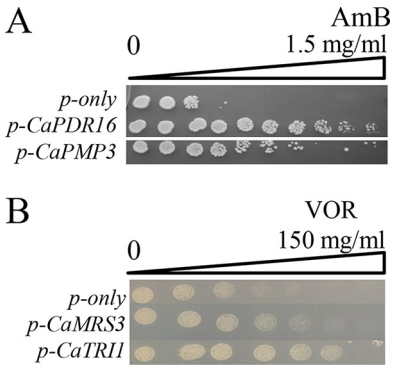

FIG 5 Test of *C. albicans* orthologous genes. *Candida* genes were cloned into yeast expression vectors and expressed in *S. cerevisiae*. (A) Cells were grown on an AmB gradient between 0 μg/ml and 1.5 μg/ml. (B) Cells were grown on a VOR gradient between 0 μg/ml and 150 μg/ml.

system. Similarly, the orthologs of the *TRI1* and *MRS3* (*CaTRI1* and *CaMRS3*) genes also provided reduced VOR susceptibility when expressed in yeast cells (Fig. 5B).

DISCUSSION

In this study, we screened a high-copy-number gDNA library to find the genes that support cell growth in the presence of lethal doses of systemically used antifungals: AmB, CSP, and VOR.

We identified the *PDR16* and *PMP3* genes for their ability to reduce AmB susceptibility. Their overexpression from a plasmid reduced susceptibility, and their deletion rendered cells susceptible to AmB treatment. Moreover, the genes were transcriptionally responsive to AmB treatment. Thus, our findings confirmed the role of *PMP3* and suggested a new gene, *PDR16*, for AmB tolerance. Both of these genes have known roles in plasma membrane lipid dynamics.

A recent study suggested that AmB binds to ergosterol and forms extramembranous and fungicidal sponge structures (21). Although AmB resistance is not common, it is observed in *Candida* spp. and other fungal species (22, 23). AmB resistance mechanisms commonly involve alterations in the cell membrane and reduction in ergosterol levels (22–25). Sphingolipids are also involved in AmB resistance. Deletion of the sphingolipid-biosynthetic genes *FEN1* and *SUR4* renders cells susceptible to AmB (26). The role of *PMP3* in AmB tolerance might be specific to AmB, since it does not provide vulnerability to AmB-related drugs, such as natamycin and filipin (13). Overexpression of *PMP3* directly or indirectly antagonizes the membrane permeation effect of AmB. A recent study showed that AmB binds to ergosterol and forms extramembranous aggregates (21). On the other hand, it is known that ergosterol biosynthesis is required for AmB susceptibility (13). A possible mechanism for Pmp3 action might be that it interferes with AmB-ergosterol interactions, which needs to be further investigated using biophysical assays.

A previous study suggested that the absence of *PMP3* hyperpolarizes the plasma membrane and sensitizes cells to salt (19). We also noticed that Δ*pmp3* cells were salt susceptible (see Fig. S1), but their membranes were not hyperpolarized. The observation of different results for the membrane potential might be due to the methods used for membrane potential prediction or to the cell background. Navarre and Goffeau used a [^{14}C]methylammonium uptake assay as the indicator of the plasma membrane potential, whereas we utilized a recently developed diS-C_3(3) method (27).

PDR16 affects ergosterol biosynthesis (28) and facilitates the movement of phosphatidylinositol between membranes (29). The drug tolerance of yeast cells overexpressing *PDR16* may be related to the alterations in membrane composition so that AmB is not able to interfere with membrane permeability. Further studies comparing the permeabilities and lipid compositions in the cellular membranes of *PDR16*-

overexpressing and *PDR16*-deficient cells may provide a better understanding of its role in drug tolerance.

Echinocandin resistance is mainly associated with the *FKS1* gene in different fungal species. Spontaneous mutations can arise in hot-spot regions of Fks1 and can reduce the enzyme's sensitivity to the drug and thus lead to drug resistance. Moreover, fungal strains have been isolated in which the sequence of *FKS1* is unaltered yet the fungus has decreased susceptibility to echinocandins (30, 31), suggesting that there are additional mechanisms governing CSP resistance. CSP was the first member of the new class of echinocandins, and we identified two novel genes, *RMD9* and *SWH1*, whose overexpression reduces susceptibility to CSP. Rmd9 has a role in the processing and stability of mRNAs encoding the subunits of the electron transport chain (ETC) (15). The *RMD9* gene was not transcriptionally activated by CSP treatment and reduced susceptibility only when expressed ectopically. The Δ*rmd9* deletion mutants are respiration deficient (15), and production of organic acids via fermentation (32) could be a reason why these cells had lower pH values. The other CSP tolerance gene that we identified was *SWH1*. The protein encoded by this gene has homology with a mammalian oxysterol binding protein (33). The primary function of Swh1 is still unknown, although it may have a role in nucleus-vacuole (NV) junction and endoplasmic reticulum (ER) trafficking as a result of its structural similarity to oxysterol binding protein (34). Mutations in the *SWH1* gene result in a reduction in membrane ergosterol levels (35). The reduced susceptibility that *SWH1* provides may be related to membrane ergosterol dynamics, membrane fusion pathways, and ER stress response (36).

Voriconazole prevents ergosterol biosynthesis by inhibiting the cytochrome P450-dependent 14-α-demethylase (CYP51) enzyme (37). Triazoles like voriconazole directly target the ferric ions of the heme group that exists in CYP51 (11). Fungi can overcome these effects by overproducing Erg11, by mutational disruption of the azole-Erg11 interaction, or by effluxing the azole from the cytoplasm (38). Voriconazole is active against many *Candida* species that are resistant to fluconazole (37). However, recent reports show that gain-of-function mutations in the *MRR1* transcription factor provide voriconazole resistance (39). In this study, the genes we found for reduced VOR susceptibility are novel genes and have not been associated with known drug resistance mechanisms previously. *MRS3* was first identified as a mitochondrial-RNA splicer (40) but was then defined as a member of the mitochondrial transport family and as responsible for iron transportation (41). In cells, ferric ions are held in the intermembrane space of the mitochondria, and the cytoplasm is free of these ions. When the cells need iron, the FeS complexes of mitochondria process iron ions and transport them to the cytoplasm (42). We found that cells significantly (17-fold) upregulate the *MRS3* gene (Fig. 2B) in response to voriconazole treatment, probably to compensate for the iron requirement. Thus, if azole drugs remove the iron ions from the CYP51 enzyme, high expression of *MRS3* may replenish the iron requirement of CYP51 and therefore could reduce drug susceptibility. *TRI1* has no known function and encodes a SUMOylated protein (18). Expression of its *Candida* homolog significantly reduced susceptibility to VOR; however, its mechanism is unknown.

The genes for CSP and VOR tolerance we have identified may be less active than the genes we found for AmB tolerance. This is especially evident when we consider the susceptibilities of the deletion mutants to the corresponding drugs. Therefore, the roles of CSP and VOR genes in drug resistance may not be physiological and may have resulted from an indirect effect of ectopic overexpression. The clinical significance of these genes should be investigated with additional experiments.

We used toxic concentrations of drugs in our gDNA screening in the hope of finding only robust genes that can reduce drug susceptibility. The doses tested here and the MIC values were in agreement with those in previous studies (43–46). We used 150 μg/ml VOR as the cutoff value for the initial screening analyses because of residual growth at high doses. This range for VOR was also observed by others for BY4741 cells (46).

In summary, our genomewide genetic screening for the identification of genes

TABLE 2 Primers used to amplify *C. albicans* (SC5314) genes

Primer	Sequence (5′–3′)
CaPDR16F	GGGGACAAGTTTGTACAAAAAAGCAGGCTCTCCACCCATACTTCTCATGTTTG
CaPDR16R	GGGGACCACTTTGTACAAGAAAGCTGGGTAGGTACATGTATCATTTTAGATGG
CaPMP3F	GGGGACAAGTTTGTACAAAAAAGCAGGCTCCACATACACCATTAATTATGAA
CaPMP3R	GGGGACCACTTTGTACAAGAAAGCTGGGTCATCAAGCAGTAAATCTTTGGTG
CaMRS3F	GGGGACAAGTTTGTACAAAAAAGCAGGCTCTATCATAATGGAGCACCAACTTC
CaMRS3R	GGGGACCACTTTGTACAAGAAAGCTGGGTTACCAAGTAGTATGATGTCTATG
CaTRI1F	GGGGACAAGTTTGTACAAAAAAGCAGGCTCAGTATTATGTCTACCATTAAGT
CaTRI1R	GGGGACCACTTTGTACAAGAAAGCTGGGTCTTATAACTCAATCCTCCTATCCC

involved in reduced drug susceptibility resulted in new potential target genes for AmB, CSP, and VOR. We limited our study to budding yeast cells as a model because of technical advantages and genetic stability. Further characterization of these genes in pathogenic fungal strains or clinical isolates may shed light on the drug resistance mechanisms and may lead to novel diagnostic tools that would allow identification of specific resistance profiles from genetic hallmarks.

MATERIALS AND METHODS

Drugs, yeast strains, cell growth and gDNA library screening. Amphotericin B (Fungizone; Bristol-Myers, Squibb), caspofungin (Sigma), and voriconazole (Vfend IV; Pfizer) were used in the library-screening, resistance, and susceptibility tests. The *S. cerevisiae* strain BY4741 (*MAT***a** *his3Δ1 leu2Δ0 met15Δ0 ura3Δ0*; EUROSCARF) and its isogenic deletion mutants were used in the study. Genomic DNA of *C. albicans* strain SC5314 (47) was used to amplify and clone the orthologous genes. A high-copy-number yeast genomic DNA library (ATCC no. 37323) was used for the screening. Yeast transformations were performed by the standard LiAc method (48). Unless otherwise indicated, all experiments were performed on YNB medium supplemented with appropriate amino acids and bases (49). YPD rich medium (1% yeast extract, 2% peptone, 2% dextrose) was also used to grow the wild type and deletion mutants before the transformation process. The *Candida* cells were grown in Sabouraud dextrose agar (SDA) medium (2% dextrose, 1% peptone, 1.5% agar, pH 5.6). Plates were incubated at 30°C for 3 days and photographed.

MIC analyses. For MIC analyses, EUCAST broth microdilution testing was performed as described in document EDEF 7.3.1 (50) with some modifications to adapt to yeast growth and screening conditions. In brief, we first prepared the yeast inoculum (5×10^6 CFU/ml) in sterile water. Instead of RPMI medium, we used YNB medium (0.67 g YNB, 2 g glucose 100 ml^{-1}) including the necessary amino acids and nucleobases (5 mg L-methionine, 10 mg L-leucine, 5 mg L-histidine, 1 mg uracil 100 ml^{-1}) for the auxotrophic requirements of the BY4741 strain. The antifungal stock solutions were prepared in 100% dimethyl sulfoxide (DMSO). Serial dilutions of antifungal drugs were prepared by using YNB medium in flat-bottom 96-well plates. After the inoculum was added, the final concentrations of antifungal drugs ranged from 0.1 to 1 mg/liter for amphotericin B, 0.0125 to 0.5 mg/liter for caspofungin, and 0.025 to 100 mg/liter for voriconazole. The plates were incubated at 30°C, and the MICs were determined spectrophotometrically at 600 nm after 24 h of incubation. The MIC_{100} for AmB and the MIC_{50} values for CSP and VOR were calculated.

Gradient plate preparation and spotting assay. Gradient plates were prepared as described by Metzenberg and Groteluesche n (51). In brief, 50 ml of agar medium without drug was poured into 120-mm by 120-mm square petri dishes and slanted at an angle of 45°. When the agar had solidified, 50 ml of the same medium including the lethal dose of the drug was poured, and the plate was left horizontal until all the agar had solidified.

For spotting assays, suspensions of fresh cells (optical density at 600 nm [OD_{600}] = 0.2) were serially diluted and dropped onto the related agar media. For gradient plates, 5 μl of cells at an OD_{600} of 0.02 were dropped one by one on the same line. The plates were incubated at 30°C for 3 days.

Plasmid isolation, sequencing, and gene cloning. Yeast cells were digested with lyticase (5 U/ml) for 30 min in Tris-EDTA (TE) buffer before the isolation. Plasmids were isolated from yeast cells using a GeneJet plasmid miniprep kit (ThermoScientific) as described by the manufacturer. The isolated plasmids were amplified using *Escherichia coli* JM109 competent cells and sequenced from both sides with a pair of vector-specific primers at the Biotechnology Center of the Izmir Institute of Technology.

The *S. cerevisiae* genes were subcloned into p425GPD (ATCC 87359) overexpression vector from the ThermoScientific OpenBiosystems Yeast ORF Collections (52). The PCR-amplified *C. albicans* genes *CaPDR16*, *CaPMP3*, *CaMRS3*, and *CaTRI1* were cloned into the pAG426GPD-ccdB destination vector by Gateway Technology (52). The primers used to amplify *Candida* (SC5314) genes are shown in Table 2. PCR conditions for the *CaPDR16*, *CaMRS3*, and *CaTRI1* genes were 94°C for 5 min, 94°C for 30 s, 46°C for 30 s, 72°C for 1.5 min, and 72°C for 7 min for 30 cycles; the conditions for *CaPMP3* were almost the same, but the annealing temperature was 45°C and the elongation time was 45 s.

Transcriptional analyses. Total RNA samples from logarithmically growing cells were isolated using a ThermoScientific GeneJet RNA purification kit. Genomic-DNA contaminations were removed by DNase treatment (DNaseRQ1; Promega).

cDNA synthesis was performed using a First Strand cDNA synthesis kit (ThermoScientific) according to the manufacturer's instructions. The cDNAs were used as templates to amplify internal parts of the selected genes. The *ACT1* gene was used as an internal control. RNA samples were obtained from two independent yeast samples, and triple reactions were set for each one. Statistical significance was evaluated with Student's *t* test. Real-time PCR assays were performed with the IQ5 real-time PCR system (Bio-Rad).

Transcriptional profiling of the whole genome was performed by microarray analysis with Agilent's single-channel array. After background correction, the data were normalized via a quantile procedure. The consistency of each sample was measured by principal-component analysis (PCA). Then, the similarities of the samples were monitored by cluster analysis. Discrimination of the varied genes was detected by fold change ($\log_2$) analysis instead of classical statistical methods. Each gene detected was classified by MIPS functional analysis using the FunSpec tools (http://funspec.med.utoronto.ca).

Relative membrane potential measurement. Relative membrane potential measurement was performed as described previously (27, 53). The wild type and deletion mutants were grown in YPD medium; strains with overexpression of the genes were grown in YNB medium, as stated above. Exponential-phase cells were harvested by centrifugation, washed twice with sterile distilled water, and resuspended in 10 mM MES (morpholineethanesulfonic acid) buffer (pH 6.0, adjusted with triethanolamine) to an OD_{600} of 0.2 for each sample. Measurements were performed in UV grade cuvettes (Kartell). The probe diS-C_3(3) was added to a final concentration of 4×10^{-8} M (10^{-5} M stock solution in ethanol). The fluorescence emission spectra (λ_{ex} [excitation wavelength] = 531 nm; λ_{em} [emission wavelength] = 560 to 590 nm) of the cell suspensions were measured with an ISS PC1 (Photon Counting) spectrofluorimeter. The staining curves recorded the dependence of the fluorescence emission maximum wavelength (λ_{max}) at the time of staining. Measurements were done with three independent biological samples.

Fluorescent imaging and cytosolic-pH measurement. Cells expressing pHluorin (transformed with the plasmid pHl-U) (54) were grown in a specific selective medium [0.175% YNB (minimal medium without ammonium sulfate, folic acid, riboflavin, and KCl; MP Biomedicals), 0.4% $(NH_4)_2SO_4$, 2% glucose, and auxotrophic supplements, including leucine, methionine, and histidine] to an OD_{600} of ~0.5.

Measurement of the cytosolic pH was based on a polynomic calibration curve generated according to the method of Orij et al. (55) and Duškova et al. (56). The pH standards were 5.67, 5.97, 6.44, 6.59, 6.80, 7.01, 7.3, and 7.76. The intensity of the fluorescence was measured in a fluorescence reader (Synergy HT; BioTek) at 400 nm and 485 nm; emission was at 516 nm. To eliminate the background fluorescence, a culture of a nontransformed strain was grown in parallel, and the corresponding values were subtracted from the fluorescence at each excitation wavelength (Gen5 software; BioTek Instruments). The I_{400}/I_{485} ratio was used to calculate the intracellular pH from the calibration curve. Each strain was measured in 8 wells (100 μl of cells per well) within one experiment (technical replicates), and the data presented are means and standard deviations (SD) of the results of two independent experiments (biological replicates).

SUPPLEMENTAL MATERIAL

Supplemental material for this article may be found at https://doi.org/10.1128/AAC.02268-18.

SUPPLEMENTAL FILE 1, PDF file, 1.7 MB.

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We declare no conflict of interest.

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