Sphingolipid Biosynthetic Pathway Genes *FEN1* and *SUR4* Modulate Amphotericin B Resistance

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Deletants of the sphingolipid biosynthetic pathway genes *FEN1* and *SUR4* of *Saccharomyces cerevisiae*, as well as deletants of their orthologs in *Candida albicans*, were found to be 2- to 5-fold-more sensitive to amphotericin B (AmB) than parent strains. The inhibition of sphingolipid biosynthesis in parent strains by myriocin sensitized them to AmB, which can be reversed by providing phytosphingosine, an intermediate in the sphingolipid pathway. These results indicate that sphingolipids modulate AmB resistance, with implications for mechanisms underlying AmB action and resistance.

Candidiasis is the fourth most common hospital-acquired infection, and it is associated with high mortality rates in cases of invasive infections (1–5). *Candida albicans* is the main causative agent, but other *Candida* species such as *C. glabrata*, *C. parapsilosis*, *C. tropicalis*, *C. krusei*, and *C. lusitanae* are becoming more prevalent (2, 6, 7). The rapid emergence of resistant strains against major antifungal drugs often renders therapy ineffective (8–10). Amphotericin B (AmB) is a commonly used fungicidal polyene drug, and resistance against it is reported mainly in some species of *Candida* (11). However, severe side effects are associated with high therapeutic doses of AmB (12). Thus, there is an urgent need for AmB analogs that have similar or higher potency than AmB but with minimal side effects. Another approach is to use AmB in combination with drugs that sensitize fungal cells to AmB or potentiate AmB action, such that less AmB is needed for effective therapy, thereby reducing AmB toxicity.

The mechanisms underlying the fungicidal actions of AmB are becoming better understood; a recently reported study (13) showed that binding of AmB to ergosterol as such is sufficient to kill the cells, and leakage of ions due to pore formation is a secondary effect of AmB. Absence of ergosterol in ergosterol biosynthesis mutants results in AmB resistance (11, 14–18). Sterols (ergosterol in fungi and cholesterol in higher eukaryotes) and sphingolipids form distinct domains (lipid rafts) in plasma membrane and are required for several cellular processes, including maintenance of plasma membrane integrity, protein sorting, endocytosis, and proper functioning of certain membrane proteins (19–24). Altered composition or loss of these membrane constituents affects targeting of ATP-binding cassette transporter Cdr1p to lipid rafts and susceptibility of *C. albicans* and *Saccharomyces cerevisiae* to drugs that are substrates of drug efflux pumps (25, 26). Since ergosterol interacts physically as well as functionally with sphingolipids, and biosynthesis of sphingolipids is closely coordinated with that of sterols (27–29), we hypothesized that sphingolipids, like ergosterol, might be involved in modulation of AmB resistance. To test this, first we evaluated AmB resistance of *S. cerevisiae* deletants impaired in sphingolipid biosynthesis. We also tested the effect of myriocin (a sphingolipid biosynthesis inhibitor) and myriocin in combination with phytosphingosine (an intermediate in the sphingolipid pathway) on the AmB resistance of wild-type strains of *S. cerevisiae* and *Candida* species.

Wild-type strains of *C. albicans*, *C. glabrata*, *C. lusitanae*, and

S. cerevisiae and deletion mutants of *S. cerevisiae* strain BY4743 and *C. albicans* strain SN95 were used in this study (Table 1). The yeast strains were regularly revived from frozen glycerol stocks on yeast extract-peptone-dextrose (YPD) agar medium (1% Bacto yeast extract, 2% Bacto peptone, 2% glucose, and 2% Bacto agar). For exponential cultures, strains were grown in YPD broth at 30°C with agitation (200 rpm). Synthetic complete (SC) agar medium plates contain a 0.17% yeast nitrogen base without amino acids and ammonium sulfate (Difco), 0.5% (wt/vol) ammonium sulfate, 2% glucose, 1.92 g/liter yeast synthetic dropout medium supplement without uracil (Sigma), 76 mg/liter uracil (Sigma), and 2% Bacto agar. RPMI 1640 medium with L-glutamine without sodium bicarbonate (Sigma) was buffered with 0.165 M morpholinepropanesulfonic acid (Sigma) to a pH of 7.0 (30). Stock solutions of AmB (2 mg/ml; Sigma), myriocin (2 mg/ml; Sigma), and phytosphingosine (10 mM; TCI chemicals) were prepared in dimethyl sulfoxide (Sigma) and stored at –20°C until use.

To test drug susceptibility by dilution spotting, the strains were grown overnight in YPD broth, reinoculated in fresh YPD medium at a starting optical density at 600 nm (OD₆₀₀) of 0.1, and incubated for 4 to 5 h at 30°C with shaking at 200 rpm until an OD₆₀₀ of 0.6 to 0.8 was reached. The cells were harvested, washed with water, and normalized to an OD₆₀₀ of 1.0 (2 × 10⁷ cells/ml). These cells were 10-fold serially diluted, and 5 µl of each dilution was spotted on SC agar plates containing different concentrations of AmB. Growth was assessed after the plates were incubated at 30°C for 2 days. We screened homozygous deletants of all nonessential genes in the sphingolipid pathway of *S. cerevisiae*, which

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TABLE 1 Strains used in this study

Strain	Description/genotype	Reference or source
<i>S. cerevisiae</i> FY4	MATa	56
<i>S. cerevisiae</i> BY4743	Mat a/α; <i>his3Δ1/his3Δ1</i> ; <i>leu2Δ0/leu2Δ0</i> ; <i>lys2Δ0/LYS2</i> ; <i>MET15/met15Δ0</i> ; <i>ura3Δ0/ura3Δ0</i>	Euroscarf
<i>C. glabrata</i> CG462	BG2, wild-type clinical isolate	57
<i>C. lusitanae</i> CL1	MATa clinical isolate	58
<i>C. lusitanae</i> CL6	MATa clinical isolate	58
<i>C. albicans</i> SC5314	Wild-type clinical isolate	59
<i>C. albicans</i> SN95	<i>arg4Δ/arg4Δ his1Δ/his1Δ</i> <i>URA3/ura3::imm⁴³⁴</i> <i>IRO1/iro1::imm⁴³⁴</i>	39
SN95F1	As SN95, <i>fen1Δ/fen1Δ</i>	This study
SN95F12	As SN95, <i>fen12Δ/fen12Δ</i>	This study
SN95F1F12	As SN95, <i>fen1Δ/fen1Δ fen12Δ/fen12Δ</i>	This study

were constructed as part of the yeast deletion project (31). Strains deleted in *SUR4* and *FEN1* were found to be hypersensitive to AmB compared to the parent strain (BY4743) by 5-fold and 2-fold, respectively (Fig. 1). These strains were confirmed as true deletants for these genes by diagnostic PCR (see Fig. S1 and 2 in the supplemental material). *FEN1* and *SUR4* are nonessential genes involved in the synthesis of very long-chain fatty acids. Other genes involved in the elongation of fatty acids are *YBR159w* and *ELO1*, which are nonessential, and *PHS1* and *TSC13*, which are essential genes (32, 33). Strains deleted in *YBR159w* and *ELO1* were not sensitive to AmB (results not shown). It has been reported that sphingolipid and ergosterol biosynthetic pathways interact genetically (27). Thus, to check if perturbation in sphingolipid levels perhaps leads to increased ergosterol levels resulting in hypersensitivity to AmB in *fen1* and *sur4* deletants, total ergosterol contents were determined in these strains (34). The ergosterol contents of the parent strain and the *fen1* deletant were 0.026% ±

0.001% and 0.031% ± 0.005% wet weight of cells, respectively, and the difference is not statistically significant (*P* = 0.0629). On the other hand, the ergosterol content of the *sur4* deletant was 0.021% ± 0.001%, and the difference with respect to the parent strain is statistically significant (*P* = 0.003). If ergosterol is the key determinant of AmB resistance, then its slight decrease in *sur4* deletant would have increased AmB resistance. On the contrary, AmB resistance is 5-fold less than that of wild type, and thus it is unlikely that ergosterol level modulates this phenotype in this deletant.

To further validate the role of *ScFEN1* and *ScSUR4* in AmB resistance, we have deleted their orthologs, *Caorf19.6343* (*CaFEN1*) and *Caorf19.908* (*CaFEN12*), respectively, in *C. albicans*. Homozygous deletion strains of these genes, as well as a strain in which both of the genes were deleted, were constructed, starting from *C. albicans* strain SN95 (Table 1). We used an *HAH2* gene deletion cassette (see Fig. S3 in the supplemental material) (GenBank accession no. KF318042) constructed in our lab for deletion of both alleles of a gene in *C. albicans* after only a single transformation. This cassette is similar to the *UAU1* cassette (35), except that instead of *URA3*, the *HIS1* marker is used, since virulence phenotypes of deletants are not reliable when *URA3* is used as a marker (36–38). On the other hand, *C. albicans* strains that are auxotrophic for histidine (or arginine) are comparable to prototrophic strains with respect to their virulence (39) and thus are not expected to confound virulence studies when used as selection markers. The *HAH2* cassette has an intact *ARG4* gene flanked with partial and nonfunctional 5' and 3' regions of the *HIS1* gene. These regions can recombine by virtue of shared direct repeats to yield a functional *HIS1* gene. The cassette is flanked with variant *Lox* sites, *LoxLE* on the left and *LoxRE* on the right. These can recombine upon expression of Cre recombinase, yielding a mutant *Lox* site that will not participate in any further recombination (40). These were introduced to evict the entire cassette after the

FIG 1 Amphotericin B susceptibility of *S. cerevisiae* (BY4743) and *C. albicans* (SN95) strains deleted in *FEN1* and *SUR4*. All strains are diploids and in deletants, both alleles of the indicated genes are deleted. *CaFEN12* is an ortholog of *ScSUR4*. Serial 10-fold dilutions of cells were spotted on synthetic complete agar plates with the indicated concentrations of AmB and incubated at 30°C for 2 days before being photographed. Comparable results were obtained with *Scfen1* and *Scsur4* deletants in an *S. cerevisiae* haploid strain (BY4741) background and in *Cafen1Δ/Δ*, *Cafen12Δ/Δ*, and double-deletant strains independently constructed in *C. albicans* strain SN95.

FIG 2 Diagnostic PCRs for *CaFEN1* (A) and *CaFEN12* (B) in parent strain (SN95) and in strains deleted in either or both of the genes. Upstream forward and downstream reverse primers, external to flanking regions of homology used for targeted gene deletion, were used for diagnostic PCR. For *CaFEN1* diagnostic PCRs, *CaFEN1*-DG-S and *CaFEN1*-DG-R1 were used, and the expected sizes of amplified products from SN95, *Cafen1Δ/Δ*, and *Cafen1Δ/Δ Cafen12Δ/Δ* strains are 1,982 bp, 880 bp, and 880 bp, respectively. For *CaFEN12* diagnostic PCRs, *CaFEN12*-DG-S and *CaFEN12*-DG-R1 were used, and the expected sizes of products from SN95, *Cafen12Δ/Δ*, and *Cafen1Δ/Δ Cafen12Δ/Δ* strains are 1,821 bp, 909 bp, and 909 bp, respectively.

desired gene deletion to recover the auxotrophic markers for reuse in additional deletion experiments.

To delete the *CaFEN1* open reading frame (ORF) (including the ORF region from -130 to $+25$ bp), the region 254 bp up-

stream of the flanking homology region, corresponding to the -384 to -131 -bp region upstream of the *CaFEN1* start codon, was PCR amplified using *CaFEN1*-US1 and *CaFEN1*-UA1 primers (see Table S1 in the supplemental material). Similarly, the downstream *CaFEN1*-flanking homology region (443 bp), corresponding to $+26$ to $+468$ bp after the *CaFEN1* stop codon, was PCR amplified with *CaFEN1*-DS2 and *CaFEN1*-DA2 primers. Both upstream and downstream *CaFEN1* flanks have 18-bp and 24-bp sequences, respectively, homologous to the *HAH2* cassette, introduced as part of the primers (see Table S1). The upstream flanking region was fused with the *HAH2* cassette by PCR with amplified upstream flanking DNA and *HAH2* as the templates and the *CaFEN1*-US1 forward primer and cassette-specific reverse primer *CaARG4*-R1130 to provide an upstream split marker. Similarly, the downstream *CaFEN1*-flanking region was fused with the *HAH2* cassette by PCR with amplified downstream flanking DNA and *HAH2* as the templates and the cassette-specific forward primer *CaARG4*-F61 and reverse primer *CaFEN1*-DA2 to provide a downstream split marker. The PCR-amplified upstream and downstream split markers were mixed together and transformed into *C. albicans* strain SN95 (*arg4Δ/arg4Δ his1Δ/his1Δ*). These would recombine *in vivo* in the 1,070-bp region shared between them and at the target locus by means of flanking homology regions. After selecting for arginine prototrophy, correct integration was confirmed by diagnostic PCRs for expected PCR product on either side of integration. For the upstream region, diagnostic PCR with the cassette-specific primer *CaHIS1*-DR and the gene-specific primer *CaFEN1*-DG-S, external (-429 to -411 of the ORF) to the upstream flanking homology region (-384 to -131 of the ORF) introduced during transformation, yielded a PCR product comparable to the expected 383-bp product. For the

FIG 3 Sensitization of wild-type cells to amphotericin B by myriocin and its reversal by phytosphingosine (PHS). *C. albicans* (SC5314), *C. glabrata* (CG462), *C. lusitanae* (CL1, CL6), and *S. cerevisiae* (FY4, BY4743) strains were tested for their sensitivity to AmB alone (top panels) or their sensitivity in the presence of myriocin (middle panels) or myriocin and PHS (lower panels). Comparable results were obtained from three independent experiments.

TABLE 2 Synergy testing of amphotericin B and myriocin

Strain	MIC ($\mu\text{g/ml}$) of AmB:		FIC	MIC ($\mu\text{g/ml}$) of myriocin:			FIC index
	Only	With myriocin		Only	With AmB	FIC	
<i>C. albicans</i> (SC5314)	0.063	0.016	0.250	2.0	0.063	0.032	0.282
<i>C. glabrata</i> (CG462)	0.125	0.016	0.125	1.0	0.125	0.125	0.250
<i>C. lusitaniae</i> (CL6)	0.250	0.063	0.250	4.0	0.250	0.063	0.313
<i>S. cerevisiae</i> (FY4)	0.125	0.032	0.250	1.0	0.063	0.063	0.313

^a MIC is defined as the concentration at which >95% of cells are killed. The synergy testing was done three times with comparable results.

downstream region, PCR with the cassette-specific forward primer CaHIS1-ter and gene-specific reverse primer CaFEN1-DG-R1, external (+520 to +540) to the downstream flanking homology region (+26 to +468 bp of the ORF), introduced during transformation gave a PCR product comparable to the expected 626-bp product (results not shown). Deletion of *CaFEN12* was carried out as described for *CaFEN1*, but using *CaFEN12*-specific primers (see Table S1 in the supplemental material).

After confirmation of the targeted deletion of one allele each of *CaFEN1* or *CaFEN12*, the heterozygous strains were grown non-selectively in YPD broth and plated on minimal plates (without arginine or histidine) to select for Arg⁺ His⁺ prototrophs. Arg⁺ His⁺ segregants arise when both alleles become deleted after spontaneous mitotic recombination (or gene conversion), followed by recombination between direct repeats within the 5' and 3' regions of *HIS1* in one of the alleles. Among the segregants, some will have the wild-type alleles of the target gene as well, which are identified by diagnostic PCR and discarded. Routinely, at least two correct segregants derived from two independent transformants are taken for further studies.

To evict the markers (after deleting the first gene), Cre recombinase, codon-optimized for expression in *C. albicans*, was used (41). For this purpose, an *MAL2*-Cre cassette (see Fig. S4 in the supplemental material) (GenBank accession number KF318043) for the expression of Cre under the control of the *CaMAL2* promoter with *SAT1* as the selection marker was constructed starting from a cassette with the *CaMET3* promoter and *URA3* marker (41). Upon transformation and selection for nourseothricin resistance, the *MAL2*-Cre cassette integrates within the *ARG4* of the *HAH2* present in one of the disrupted alleles, by virtue of flanking homology to *ARG4*. The Cre recombinase, upon induction with maltose, recombines *LoxLE* and *LoxRE* sites flanking the markers. The resulting clones that were auxotrophic to histidine and arginine and sensitive to nourseothricin were tested for true deletion of *CaFEN1* or *CaFEN12* gene by diagnostic PCR (Fig. 2). *CaFEN12* was deleted in a *Cafen1Δ/Δ* strain, as described above, to yield double-deletant strains (*Cafen1Δ/Δ* *Cafen12Δ/Δ*). The selection markers were also evicted from this strain to make the auxotrophies comparable in all the strains. The deleted *CaFEN1* and *CaFEN12* regions were PCR amplified and sequenced to further confirm the deletions and proper evictions of markers after recombination at *Lox* sites (see Fig. S5 and S6 in the supplemental material).

The sensitivities of *Candida* deletants to AmB were checked by dilution spotting. The *Cafen1Δ/Δ* and *Cafen12Δ/Δ* deletants were 2-fold- and 5-fold-more sensitive than the parent strain (Fig. 1), similar to the sensitivities of the deletants of *S. cerevisiae* orthologs. We could construct a *C. albicans* strain deleted in both these genes as well, though double deletion of *ScFEN1* and *ScSUR4* is syn-

etically lethal in *S. cerevisiae* (42). The *Cafen1Δ/Δ* *Cafen12Δ/Δ* double deletant was slow growing and at least 10-fold more sensitive to AmB than the parent strain SN95 (Fig. 1), confirming the role of these genes in modulating AmB resistance.

Since deletion of only two genes in the sphingolipid biosynthetic pathway sensitized cells to AmB, we tested the effects on AmB resistance of chemical inhibition of sphingolipid biosynthesis in wild-type strains. Myriocin is a specific inhibitor of serine palmitoyltransferase encoded by the essential genes *LCB1* and *LCB2*, which catalyze the first committed step of sphingolipid biosynthesis (43, 44). We incubated *C. albicans* (SC5314), *C. glabrata* (CG462), *C. lusitaniae* (CL1 and CL6), and *S. cerevisiae* (FY4 and BY4743) strains at different concentrations of AmB and myriocin, alone and in combination. Myriocin at a sublethal concentration (0.4 $\mu\text{g/ml}$) rendered the cells sensitive to sublethal concentrations of AmB (0.1 $\mu\text{g/ml}$ for *C. albicans*, *S. cerevisiae*, and *C. lusitaniae* strain CL1 and 0.25 $\mu\text{g/ml}$ for *C. glabrata* and *C. lusitaniae* strain CL6), indicating that inhibition of sphingolipid biosynthesis sensitizes cells to AmB (Fig. 3). To quantitate this, a checkerboard broth microdilution assay using a CLSI standard reference method (30) was used. Myriocin sensitized cells to AmB by 4- to 8-fold and was synergistic with AmB for all the tested strains, with a fractional inhibitory concentration (FIC) index of <0.5 (Table 2). However, the synergistic effect of myriocin remains to be verified with other pathogenic *Candida* species. To confirm if AmB sensitivity is due to depletion of sphingolipids, we supplemented AmB-myriocin plates with a sublethal concentration (10 μM) of phytosphingosine (PHS). PHS is an intermediate downstream of serine palmitoyltransferase in sphingolipid biosynthesis, and it is known to rescue myriocin inhibition of sphingolipid biosynthesis (45). We found that PHS reversed the AmB sensitivity of myriocin-treated cells, confirming the role of sphingolipids in AmB resistance (Fig. 3).

To the best of our knowledge, this is the first study demonstrating the role of sphingolipid pathway genes in modulating AmB resistance of *S. cerevisiae* and *Candida* species. *FEN1* (also known as *ELO2*, *GNS1*, and *VBM2*) and *SUR4* (also known as *ELO3*, *SRE1*, *VBM1*, and *APA1*) encode fatty acid elongases. These enzymes are part of very long-chain fatty acid synthetase complexes, which are involved in the synthesis of C₂₂ and C₂₄ (Fen1p) or C₂₆ (Sur4p) fatty acids (46, 47). *FEN1* and *SUR4* deletants show several pleiotropic phenotypes, including defects in secretory pathways, lipid rafts, and vacuolar ATPase activities (48–51). Detailed comparative lipidomics analysis has shown that deletion of *FEN1* or *SUR4* perturbed the entire yeast lipidome (52). Such changes, thought to be due to unrelated compensatory “ripple effects” (52), may be responsible for the pleiotropic phenotypes seen with the deletion of individual genes. *S. cerevisiae* *sur4* and *fen1* deletants overexpressing *C. albicans* drug efflux pump Cdr1p were found to

be sensitive to azole and other drugs, which are substrates of Cdr1p (26). This sensitivity was attributed to mislocalization and improper functioning of Cdr1p due to membrane lipid imbalance. This is unlikely to explain sensitivity of these deletants to AmB since it is not a substrate of drug efflux pumps. We have checked if AmB sensitivity of these deletants is modulated by any compensatory changes in ergosterol levels, but did not find any correlation with ergosterol content. However, since ergosterol is preferentially associated with sphingolipids in lipid rafts (53, 54), depletion of sphingolipids may render ergosterol more accessible to AmB binding, thereby sensitizing cells to AmB. While this appears to be a straightforward possibility, depletion of sphingolipids might also affect AmB resistance indirectly; since ergosterol and sphingolipids are critical for several cellular functions, impairment of one or more of these functions may sensitize cells to AmB. Experiments are under way to unravel the mechanism(s) through which Fen1p/Sur4p and sphingolipids modulate AmB resistance.

Recently, Healey et al. (55) reported that strains deleted in *FEN1* and *SUR4* show reduced susceptibility to caspofungin but increased susceptibility to micafungin. This is rather paradoxical since both these drugs are echinocandins, and the possible mechanism for differential susceptibility could only be speculated upon (55). Myriocin treatment sensitized cells to both caspofungin and micafungin (55), confirming the importance of sphingolipids in modulating antifungal resistance. Myriocin is an immunosuppressor and quite toxic to humans and thus cannot be used in combination with AmB or echinocandins. Nevertheless, nontoxic analogs of myriocin, if developed, can be used to sensitize pathogenic fungi to these antifungals, thereby enhancing their therapeutic efficacy.

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