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Identification of the *FKS1* Gene of *Candida albicans* as the Essential Target of 1,3-β-D-Glucan Synthase Inhibitors

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Pneumocandins and echinocandins are fungicidal antibiotics, currently in clinical development, that inhibit 1,3-β-D-glucan synthase (GS) in several human fungal pathogens. We have identified a gene from the diploid organism *Candida albicans* that encodes a target of these inhibitors. A 2.1-kb portion of this gene, designated *CaFKS1*, has significant homology to the *Saccharomyces cerevisiae FKS1* and *FKS2* genes, which encode partially functionally redundant subunits of GS. To evaluate the role of CaFks1p in susceptibility to echinocandins, we disrupted *CaFKS1* on one homolog each of the spontaneous pneumocandin-resistant *C. albicans* mutants CAI4R1, NR2, NR3, and NR4. These mutants had been selected previously on agar plates containing the pneumocandin L-733,560. The clones derived from this transformation were either resistant (Ech^r) or fully sensitive (Ech^s) to inhibition by L-733,560 in both liquid broth microdilution and in vitro GS assays. The site of plasmid insertion in the transformants was mapped by Southern blot analysis, using restriction site polymorphisms in the *CaFKS1* gene to distinguish between the two alleles (designated *CaFKS1h* and *CaFKS1b*). For strains CAI4R1 and NR2, the *CaFKS1b* allele was disrupted in each Ech^r transformant; for strain NR4, *CaFKS1h* was disrupted in each Ech^r transformant. We conclude that (i) strains CAI4R1, NR2, and NR4 are heterozygous for a dominant or semidominant pneumocandin resistance mutation at *CaFKS1*, (ii) drug resistance mutations can occur in either *CaFKS1* allele, and (iii) CaFks1p is a target of the echinocandins. For transformants of strain NR3, all the clones we analyzed were uniformly Ech^r, and only the *CaFKS1h* allele, either in disrupted or wild-type form, was detected on genomic Southern blots. We believe gene conversion at the *CaFKS1* locus may have produced two *Cafks1h* alleles that each contain an Ech^r mutation. Transformants derived from the mutants were analyzed for susceptibility to pneumocandin treatment in a mouse model of disseminated candidiasis. Strains heterozygous for the resistant allele (i.e., *C. albicans* CAI4R1, NR2, and NR4) were moderately resistant to treatment, while strains without a functional Ech^s allele (i.e., strain NR3 and derivatives of strain CAI4R1 with the disruption plasmid integrated in the Ech^s allele) displayed strong in vivo echinocandin resistance. Finally, we were unable to inactivate both alleles at *CaFKS1* by two-step integrative disruption, suggesting that CaFks1p is likely to be an essential protein in *C. albicans*.

Semisynthetic derivatives of pneumocandin B_0 (MK0991; formerly L-743,872) and echinocandin B (LY303366) are lipopeptide antifungals that are currently in clinical trials for the treatment of systemic fungal infections (5, 33). These compounds inhibit the synthesis of 1,3-β-D-glucan, which is an essential polysaccharide in the walls of many, if not all, fungal pathogens (3, 10, 18). The broader-spectrum semisynthetic derivatives have increased potency in vitro and in animal models of fungal infection, and the improved potency is a function of increased activity against 1,3-β-D-glucan synthase (GS) (3, 30). Despite widespread interest in this enzyme target for antifungal therapy, many questions remain about the components of the synthetic complex, the mechanism of echinocandin inhibition, and the potential for resistance in the fungal pathogens.

The susceptibility of *Saccharomyces cerevisiae* to GS inhibitors has been used to identify and characterize genes that play a role in 1,3-β-D-glucan synthesis (9, 11, 12, 15). Based on studies of resistant mutants (9, 11, 12, 15), gene disruption experiments (11, 22, 37), and biochemical characterization, the following model has emerged. *S. cerevisiae* has two genes, *FKS1* and *FKS2*, which encode highly homologous, large integral membrane proteins that function as the catalytic subunit of GS (11, 22, 37). A small GTP-binding subunit, Rho1p, is also necessary for activity (13, 20, 24, 36, 41). *FKS1* expression is cell cycle regulated and more abundant during vegetative growth, while *FKS2* expression is calcineurin dependent and important for efficient sporulation (37). Mutations conferring high-level resistance to lipopeptides (the Ech^r phenotype) map to *FKS1* or *FKS2*, and the GS complexes containing either Fks1p or Fks2p are sensitive to inhibition by lipopeptides (11, 37). The mutant alleles confer resistance to GS inhibitors, both in whole cells and in assays of GS activity, and the resistance phenotype is semidominant in diploids. The role for Fks1p in glucan synthesis was further supported by Inoue et al. (22), who identified the gene products of *FKS1* and *FKS2* in enzyme preparations enriched for GS activity. As would be expected for an essential cellular function, mutants can survive with either *FKS1* or *FKS2* alone but disruption of both genes is lethal (22, 37).

By using a portion of *S. cerevisiae FKS1* as a probe, genes with strong homology were identified from the fungal pathogens *Candida albicans*, *Aspergillus fumigatus*, and *Cryptococcus neoformans* (11). The homolog *fksA* has recently been cloned from *Aspergillus nidulans* (25). The deduced amino acid sequence for FksAp is significantly conserved with the *S. cerevi-*

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siae Fks proteins, particularly in a central domain which is predicted to be hydrophilic and on the cytoplasmic face of the protein. To explore the role of FksAp in glucan synthesis, Kelly et al. (25) raised antisera against synthetic peptides designed from the FksAp sequence and demonstrated that a cross-reacting protein with the expected molecular weight was detected and strongly enriched during partial purification of GS from *A. nidulans*. This enzyme activity was susceptible to lipopeptide inhibition, and the hyphae of *Aspergillus* spp. become distended and highly branched, both in vitro (32) and in vivo (28), when exposed to GS inhibitors. The results support the idea that FksAp is both a component of the GS complex in *A. nidulans* and a target of the echinocandins.

A recent in vitro selection for spontaneous L-733,560-resistant mutants of the *C. albicans* Ura$^-$ strain CAI4 yielded rare isolates whose phenotypes were similar to those of *S. cerevisiae* lipopeptide-resistant mutants (27). The whole-cell resistance of one such mutant (strain CAI4R1) reflects resistance in enzyme activity, and results from spheroplast fusion experiments are consistent with a semidominant mutation in a single gene (27). As with the *S. cerevisiae* mutants, the resistance phenotype is specific to GS inhibitors. To understand the nature of echinocandin resistance in these spontaneous mutants, and to explore the role of the *C. albicans* homolog of *FKS1* in susceptibility to GS inhibitors, we cloned and sequenced a portion of the homologous gene (*CaFKS1*) and used it for integrative disruption in several *C. albicans* Ech^r mutants. Our results indicate that mutations in either *CaFKS1* allele can confer in vitro resistance to L-733,560, which identifies CaFks1p as a target of the echinocandins in *C. albicans*. All of the transformants retained the ability to produce a lethal infection in mice, and disruption of the Ech^s allele correlated with significant resistance to treatment of infected animals with L-733,560. The absence of DNA highly homologous to *CaFKS1* on Southern blots of *C. albicans* total genomic DNA, along with our inability to recover null mutations in both *CaFKS1* alleles, suggests that there may be no functional redundancy for this gene in *C. albicans*. The clinical implications of these results are discussed.

MATERIALS AND METHODS

Strains, media, antibiotics, and growth conditions. *C. albicans* CAI4 (16) was a gift from W. Fonzi (Georgetown University). The Ech^r mutants CAI4R1, NR2, NR3, and NR4 have been described previously (27). The semisynthetic pneumocandin L-733,560 (6) was synthesized at Merck (Rahway, N.J.) and was shown to be >95% pure by high-performance liquid chromatography. Amphotericin B, itraconazole, ketoconazole, nystatin, and 5-fluorocytosine were obtained from Sigma. Fluconazole was from the Merck chemical collection. Strains were grown at 30°C in yeast extract-peptone medium with adenine, with or without uridine supplementation (YPDA and YPDAUd, respectively), or in a defined medium (SC Ura$^-$) as described previously (27). Values for the MIC or for 50% inhibition of growth by L-733,560 were determined by a liquid broth microdilution assay in either YPDA, YPDAUd, or Yeast Nitrogen Base (Difco) with 2% glucose. Briefly, cells grown to mid-logarithmic phase were subcultured to an initial optical density at 600 nm (A_{600}) of 0.01 and seeded in wells of microtiter dishes that contained serial twofold dilutions of L-733,560. Growth inhibition was determined after 24 h at 30°C by eye (for MICs) or by measuring the A_{600} in a spectrophotometer (Tecan) and comparing the results to those for a control well containing no drug.

DNA and RNA methods. All DNA manipulations were performed as described by Maniatis et al. (34). Wizard Preps (Promega) and kits from Qiagen, Inc., were used to isolate plasmids, and DNA fragments were purified with the Gene Clean Kit (Bio101). Enzymes for digestion, ligation, and dephosphorylation of DNA were from Gibco BRL. Competent cells of *Escherichia coli* DH5α were used for transformations.

Total genomic DNA was isolated from *C. albicans* strains by the method of Holm et al. (21). For Southern blot analysis, probes prepared by random priming (Stratagene) with [^{32}P]dCTP (Amersham) were hybridized to DNA that had been digested with restriction enzymes and transferred to Zeta Probe GT membranes (Bio-Rad) with a PosiBlot pressure blotter (Stratagene) according to the manufacturer's directions. Blots were washed under conditions of high stringency and analyzed by autoradiography.

DNA was transformed into *C. albicans* by either the spheroplast method (29) or the lithium acetate method (14). Transformants selected on SC Ura$^-$ medium were purified and tested for growth on YPDA agar plates containing L-733,560 at 0.5 μg/ml.

DNA sequence analysis was performed as per the manufacturer's directions, using Sequenase v. 2.0 (U.S. Biochemicals), [^{32}P]dCTP (Amersham) and oligonucleotide primers synthesized at Merck or purchased from New England Biolabs or Gibco BRL. Software from Mac Vector (Oxford Molecular Group) and the Genetics Computer Group was used to analyze DNA sequences.

Northern blotting was performed according to the manufacturer's directions, using mRNA prepared with the polyATract System (Promega). Samples were separated on a 1% agarose gel containing formaldehyde, transferred to a nylon membrane (Amersham), and probed with a DNA fragment radiolabeled with [^{32}P]dCTP by random priming.

Plasmid constructions. Total genomic DNA from *C. albicans* CAI4 was digested with *Kpn*I and *Bam*HI, and fragments were separated on a 0.6% agarose gel. DNA from one lane was probed for fragments homologous to a 1.3-kb *Cla*I-*Sal*I probe from the *S. cerevisiae FKS1* gene (11). We used the signal from the Southern blot as a guide to excise *Kpn*I-*Bam*HI fragments of the appropriate size from the gel and clone them into YEplac195 (17). *E. coli* clones transformed with this minilibrary were pooled and probed with the μWave colony hybridization kit (Invitrogen). Plasmid DNA was isolated from putative clones, and the insert was identified by Southern blotting. The 2.1-kb *Kpn*I-*Bam*HI fragment from one of these clones (pGJ1) was isolated and subcloned into pUC19 (New England Biolabs) to yield pJAD1.

Plasmid pJAD1DXH was derived from plasmid pJAD1 by ligating filled-in *Xba*I and *Hin*dIII sites to yield a vector with a unique *Sal*I site within the insert DNA. pJAD1DXH was digested with *Bam*HI and phosphatase and ligated to the *URA3* gene carried on a 2.9-kb *Bam*HI fragment from plasmid pMB-7 (16) to create plasmids pAM600 and pAM601, which differ only in the orientation of the *URA3* fragment. The disruption plasmid pAM602 was created by removing the 0.6-kb *Sal*I-*Bcl*I fragment from plasmid pJAD1DXH and replacing it with the 3.9-kb *Sal*I-*Bgl*II fragment from plasmid pMB-7, which carries the *hisG-URA3-hisG* cassette.

Plasmid pJAM15 (35), with the *C. albicans URA3* gene inserted into vector pBR322, was used to transform strains CAI4, CAI4R1, NR2, NR3, and NR4 to uridine prototrophy.

GS assays. We measured the in vitro synthesis of 1,3-β-D-glucan from UDP-[^{3}H]glucose, using crude membranes prepared as described previously (12). The assay measures the formation of radiolabeled trichloroacetic acid (TCA)-insoluble material. Briefly, cells in logarithmic phase were harvested by centrifugation, washed, and broken with glass beads. Debris and unbroken cells were removed by centrifugation at 4,000 × *g*, and crude membranes were harvested from the supernatant fraction by centrifugation at 100,000 × *g*. The resulting pellet was resuspended at 1/50 the original culture volume and stored in aliquots at −80°C. For the GS assay, reaction mixtures incubated in microtiter dishes with gentle agitation for 2 h were quenched with an equal volume of ice-cold 20% TCA. Precipitated product was harvested onto glass-fiber filter mats (Pharmacia) and washed with water in a 96-channel cell harvester (Cambridge Technology). The mats were dried, sealed in bags with scintillation cocktail, and counted with a Betaplate scintillation counter (LKB/Wallac). The counting efficiency was estimated at 40%.

In vivo analysis. Uridine prototrophic derivatives of wild-type and mutant strains were tested as described previously (27) for virulence in mice. The 50% lethal doses (LD_{50}s) were calculated by the Knudsen-Curtis method (26). Strains were also tested for susceptibility to L-733,560 or amphotericin B treatment in a disseminated candidiasis target-organ assay described previously (4). The 99% effective dose (ED_{99}) was determined based on the reduction of the number of CFU/gram of kidney compared to sham-treated control mice as described previously (2). Five mice were used per group per experiment. All procedures were performed in accordance with the highest standards for the humane handling, care, and treatment of research animals and were approved by the Merck Institutional Animal Care and Use Committee. The care and use of research animals at Merck meet or exceed all applicable local, national, and international laws and regulations.

Nucleotide sequence accession number. The nucleotide sequence of the 2,069-bp *Kpn*I-*Bam*HI fragment of *CaFKS1* has been assigned GenBank accession no. AF027295.

RESULTS

A *C. albicans* homolog of *S. cerevisiae FKS1*. The Fks proteins from *S. cerevisiae* (Fks1p and Fks2p) and *A. nidulans* (FksAp) are large integral membrane proteins that share a highly homologous internal domain (domain I) which is predicted to be on the cytoplasmic side of the plasma membrane. Regions within domain I have homology to BcsAp, the catalytic subunit of *Acetobacter xylinium* cellulose synthase (25). We used the 1.3-kb *Cla*I-*Sal*I DNA fragment from *FKS1*, which encodes a

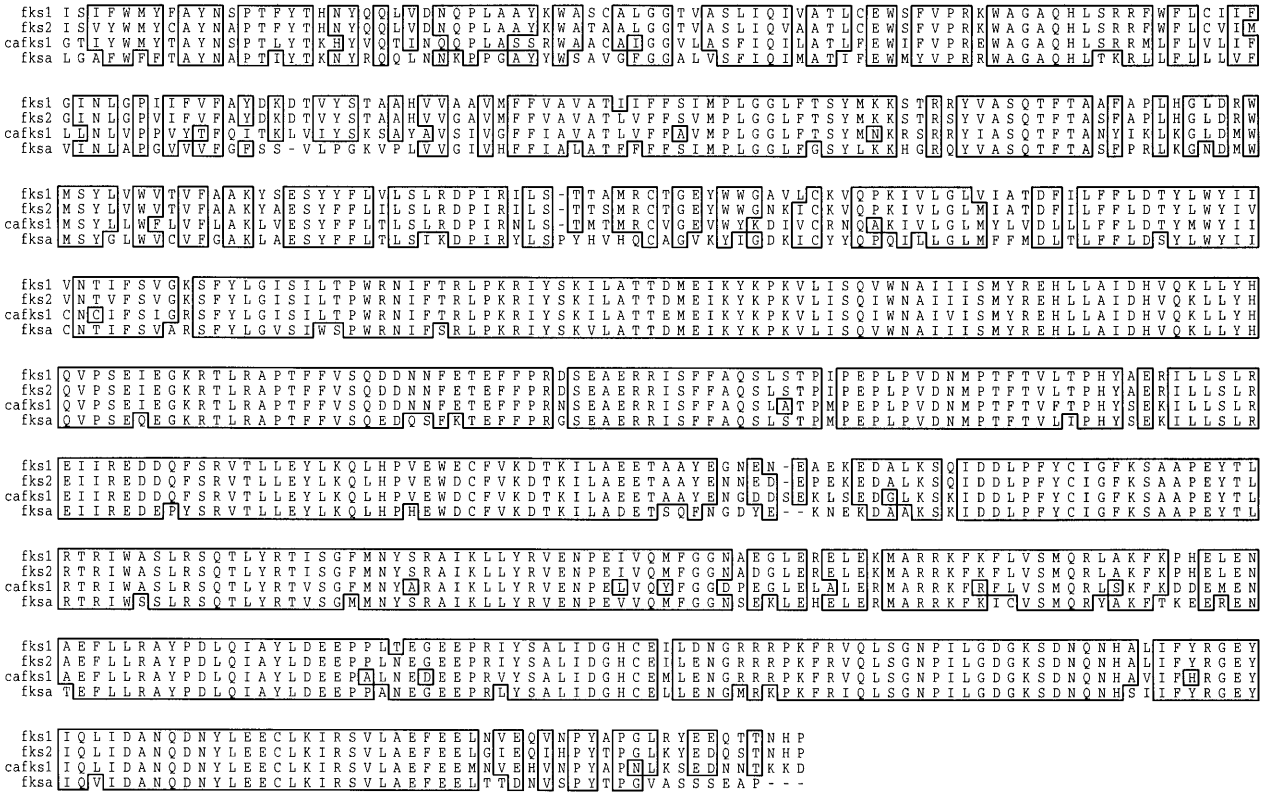

FIG. 1. Alignment of CaFks1p and predicted Fks protein sequences from *S. cerevisiae FKS1* (U12893), *S. cerevisiae FKS2* (U16783), and *A. nidulans fksA* (U51272). GenBank accession numbers are given in parentheses. The 2,069-bp *Kpn*I-*Bam*HI *CaFKS1* fragment from *C. albicans* CAI4 was analyzed by using software from the Genetics Computer Group. A 689-amino-acid uninterrupted reading frame was identified and aligned with other Fksp sequences by using the clustal program. Domain I of *S. cerevisiae* Fks1p begins at position 261 in this figure and extends beyond position 688. Conserved residues are boxed, and gaps are indicated with dashes. The regions shown in the alignment are as follows: *S. cerevisiae* Fks1p, residues 460 to 1147; *S. cerevisiae* Fks2p, residues 479 to 1166; and *A. nidulans* Fks1p, residues 492 to 1178.

portion of the large cytoplasmic domain, to clone a *Kpn*I-*Bam*HI fragment of the homologous gene from *C. albicans* and create plasmid pJAD1. As expected, the protein sequence of the uninterrupted reading frame predicted from this fragment shows strong homology to Fks1p, Fks2p, and FksAp, particularly within domain I (Fig. 1). A Northern blot of poly(A) RNA from *C. albicans* CAI4 was probed with the *Kpn*I-*Bam*HI fragment from pJAD1, and a single transcript of ca. 7 kb was detected (data not shown). We have chosen *FKS1* as the name for the open reading frame that contains this *Kpn*I-*Bam*HI fragment, given the significant degree of homology to members of the *FKS* gene family and the convention of using the same nomenclature for *C. albicans* and *S. cerevisiae* genes. We refer to it here as *CaFKS1* to avoid confusion with the *S. cerevisiae* gene.

We used the *CaFKS1* fragment in pJAD1 to map the gene in strain CAI4 by Southern blotting. The region between the *Kpn*I and *Bam*HI sites of the plasmid clone and the genome were colinear. Digestion of genomic DNA from strain CAI4 with several enzymes for which there were no sites within the *Kpn*I-*Bam*HI fragment of pJAD1 produced only a single cross-hybridizing band on autoradiograms; no additional fragments with weak homology to the probe were visible (data not shown). However, digestion of genomic DNA with *Hin*dIII and *Pvu*II generated two distinct fragments on Southern blots (Fig. 2A, lane 1), despite the fact that there were no sites for these enzymes within the *Kpn*I-*Bam*HI *CaFKS1* fragment in pJAD1. The fragment pattern was consistent with a polymorphism between the two *CaFKS1* alleles at a *Hin*dIII site in the 5′ region (Fig. 2B). We used this restriction fragment length polymorphism to distinguish the alleles in subsequent experiments. For our purposes, the 6.7-kb *Hin*dIII-*Hin*dIII fragment produced by digestion of genomic DNA from strain CAI4 with *Hin*dIII and *Pvu*II defines the *CaFKS1h* allele, while the 8.5-kb *Pvu*II-*Hin*dIII fragment defines the *CaFKS1b* allele.

Integrative transformation at the *CaFKS1* locus of Ech^r mutants. Strain CAI4R1 is a spontaneous Ech^r mutant derived from strain CAI4 as described previously (27). We used the cloned fragment of *CaFKS1* to characterize the mutation in CAI4R1. Plasmid pAM600 (Fig. 2C), which contains the *C. albicans URA3* gene, an internal fragment of *CaFKS1*, and the pUC19 backbone, was linearized within the *CaFKS1* insert by digestion with *Sal*I and transformed into CAI4R1. Integration at the *Cafks1-1* locus is expected to disrupt one of the two alleles according to the scheme outlined in Fig. 2D and E. Five of the eight Ura$^+$ transformants we characterized were Ech^r, while the other three were Ech^s. We mapped the site of integration for two Ech^r isolates (T25 and T26) and two Ech^s isolates (T28 and T32) by Southern blotting. In Fig. 2A, the two alleles at the *CaFKS1* locus of strains CAI4 and CAI4R1 and the transformants are distinguished by digestion with *Pvu*II and *Hin*dIII. For transformants T25 and T26, the 6.7-kb *Hin*dIII-*Hin*dIII fragment is intact but the 8.5-kb *Pvu*II-*Hin*dIII fragment is replaced by two novel fragments of 7.9 and 6.1 kb. Transformants T28 and T32 retained the 8.5-kb *Pvu*II-*Hin*dIII fragment, but the 6.7-kb *Hin*dIII-*Hin*dIII frag-

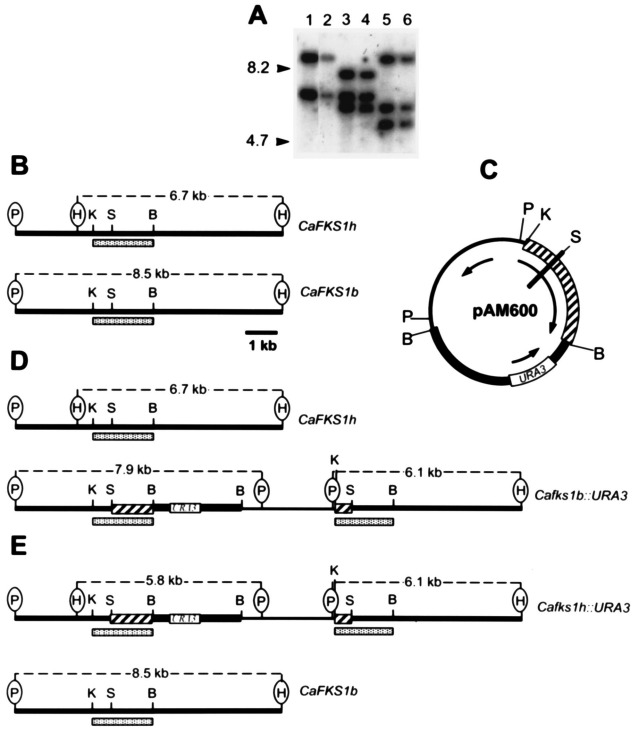

FIG. 2. Integration of plasmid pAM600 at the *CaFKS1* locus of *C. albicans* CAI4R1. (A) Southern blot of pAM600 transformants. Genomic DNAs from a wild-type strain (CAI4 [lane 1]), the original Ech^r mutant strain (CAI4R1 [lane 2]), two Ech^r transformants (T25 [lane 3] and T26 [lane 4]), and two Ech^s transformants (T28 [lane 5] and T32 [lane 6]) were digested with *Pvu*II and *Hin*dIII and probed with the 2.1-kb *Kpn*I-*Bam*HI *CaFKS1* fragment from pJAD1. A portion of the autoradiogram is shown; arrows indicate the migration positions of plasmid pAM602 digested with *Kpn*I (8.2 kb) and plasmid pJAD1DXH digested with *Bam*HI (4.7 kb). (B) Schematic maps of the *CaFKS1h* and *CaFKS1b* alleles. Abbreviations for restriction enzyme cleavage sites are as follows: P, *Pvu*II; H, *Hin*dIII; K, *Kpn*I; S, *Sal*I; and B, *Bam*HI. The K, S, and B sites are included for reference only and should not be considered unique within the regions shown. Restriction fragments detected following digestion with *Pvu*II and *Hin*dIII (circled) and probing with the *Kpn*I-*Bam*HI *CaFKS1* fragment are illustrated with dashed lines. The stippled boxes depict the regions homologous to the probe. (C) Map of plasmid pAM600. The pUC19 vector sequences (▬), the 2.9-kb *Bam*HI fragment (▬) containing the *C. albicans URA3* gene, and the 2.1-kb *CaFKS1 Kpn*I-*Bam*HI fragment (▨) are shown. Arrows within the circle illustrate the directions of transcription of the *URA3*, *CaFKS1*, and *E. coli bla* genes. The plasmid was linearized by digestion with *Sal*I (thick line intersecting the circle) prior to transformation. (D and E) Chromosomal maps following pAM600 integration at *CaFKS1*. The maps depict pAM600 integration at the *CaFKS1b* allele (D) and at the *CaFKS1h* allele (E).

ment has been replaced by novel fragments of 6.1 and 5.8 kb. These fragment sizes are consistent with integration of pAM600, which has two internal *Pvu*II sites, into the *CaFKS1b* allele of T25 and T26 and the *Cafks1h-1* allele of T28 and T32 (Fig. 2D and E).

To test the phenotypes of *Cafks1*::*URA3*/*CaFKS1* transformants T25, T26, T28, and T32, we performed a liquid broth microdilution assay and an in vitro GS assay (Fig. 3). Estimated values for 50% inhibition of growth by L-733,560 (Fig. 3A) were roughly 1,000-fold higher for transformants T25 and T26 (ca. 3,000 ng/ml) than for strains T28 and T32 (ca. 0.2 to 0.8 ng/ml). The original heterozygous mutant (CAI4R1) was also resistant, albeit to a slightly lesser extent. Figure 3B shows the curves for L-733,560 inhibition of GS in crude membrane preparations from each strain. Much like the results with whole cells, the concentrations of L-733,560 that inhibit GS activity by 50% (IC_{50}s) for strains T25 and T26 were ca. 1,000-fold higher than the values for T28 and T32. It was shown previously that the inhibition curve for CAI4R1 was unlike the titration curves described for *S. cerevisiae* Ech^r mutants or for the *C. albicans* Ech^r mutant strain CA-2 (27). The IC_{50} of L-733,560 for the enzyme from strain CAI4R1 was increased only fourfold relative to that from strain CAI4, but inhibition did not exceed 55%, even at the highest concentrations of drug we tested (27).

We used disruption with pAM600 to characterize the *Cafks1* alleles in three additional spontaneous L-733,560-resistant mutants derived from *C. albicans* CAI4: strains NR2, NR3, and NR4 (27) (Table 1). *C. albicans* NR2 and NR4, like strain CAI4R1, yielded both Ech^r and Ech^s isolates when transformed with pAM600. Strain NR2 was equivalent to CAI4R1 in that the *CaFKS1b* allele was disrupted in all Ech^r Ura$^+$ transformants, and the *Cafks1h* allele was disrupted in all Ech^s Ura$^+$ transformants (data not shown). In contrast, when strain NR4 was transformed with pAM600, loss of the resistance

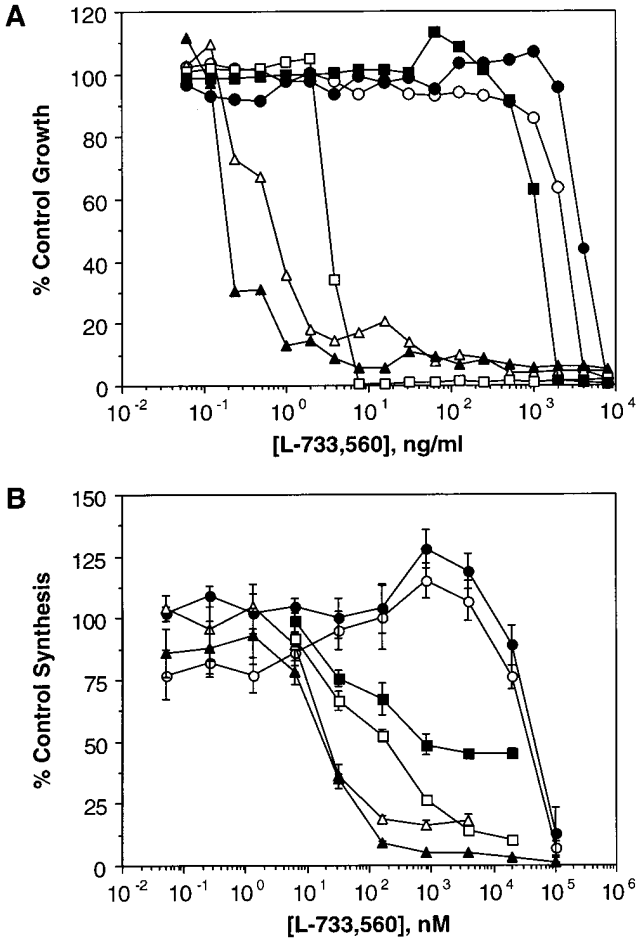

FIG. 3. Susceptibilities of CAI4R1 transformants to pneumocandin L-733,560. (A) Strains T25 (○), T26 (●), T28 (△), T32 (▲), CAI4 (□), and CAI4R1 (■) were grown in YPDA and assayed as described in Materials and Methods. The drug was prepared from a 10-mg/ml stock, and the final concentration ranged from 6.1×10^{-2} to 8.0×10^3 ng/ml. (B) Membranes were prepared from mid-logarithmic-phase cells and glucan synthase activity was measured as described in Materials and Methods. The reaction mixtures contained 0.3 mg of crude membrane protein per ml and 0.6 mM UDP-glucose with UDP-[^{3}H]glucose added to 25,000 dpm/nmol; final concentrations of L-733,560 were from 5.1×10^{-2} to 1.0×10^5 nM. After 2 h at 22°C, the TCA-insoluble product was harvested and radioactive counts were performed. The specific activity for each enzyme is given in Table 3. The symbols in panel B are the same as those used in panel A; error bars show standard deviations.

TABLE 1. Susceptibilities of CAI4R1 and pAM600 transformants of spontaneous Ech^r mutants NR2, NR3, and NR4

Strain	Susceptibility to L-733,560		No. of transformants		Disrupted allele in Ech^r transformants
	MIC[a] (μg/ml)	IC_{50} (nM)	Ech^r	Ech^S	
CAI4R1	2	1,000	5	3	*CaFKS1b*
NR2	4	40,000	5	3	*CaFKS1b*
NR3	>16	30,000	75	0	
NR4	8	40	6	2	*CaFKS1h*

[a] The MIC was determined in YPDA medium and measured as outlined in Materials and Methods.

phenotype among four Ura$^+$ transformants correlated with disruption of the *Cafks1b* allele (data not shown). These data suggest that strains NR2 and NR4, like mutant CAI4R1, are heterozygous for a semidominant mutation conferring the Ech^r phenotype and that one allele (*Cafks1h*) contains the mutation in strains NR2 and CAI4R1 while the other allele (*Cafks1b*) contains the mutation in strain NR4. With strain NR3, no Ech^s clones were recovered among 75 Ura$^+$ pAM600 transformants; i.e., all the isolates grew on SC Ura$^-$ plates containing 0.5 μg of L-733,560 per ml. The *CaFKS1* locus from three NR3(pAM600) clones and the original NR3 mutant was evaluated by Southern blotting, and the results are shown in Fig. 4A. Restriction fragments of 5.8, 6.1, and 6.7 kb were detected in *Pvu*II-*Hin*dIII-digested genomic DNA from the transformants, and a single 6.7-kb fragment was present in the digest of NR3 DNA. The faint thin line above this fragment is an artifact from film development that was present in several lanes of the autoradiogram (data not shown). These results are consistent with the genomic maps shown in Fig. 4B and C, which depict a *CaFKS1* locus with a 5′ *Hin*dIII site in both alleles (strain NR3) (Fig. 4B), and pAM600-mediated integrative disruption at this locus (Fig. 4C).

The inhibition of GS activity from strains NR2, NR3, and NR4 by L-733,560 is shown in Fig. 5. Relative to GS activity from the wild-type strain CAI4, the enzyme from each mutant

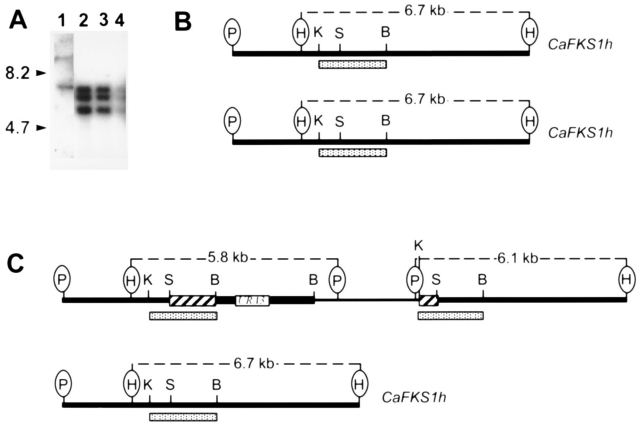

FIG. 4. *CaFKS1* locus in Ech^r transformants of mutant NR3. (A) Southern blot of DNAs from mutant strain NR3 (lane 1) and three pAM600 transformants (strains T82 [lane 2], T83 [lane 3], and T85 [lane 4]) digested with *Pvu*II and *Hin*dIII and probed with the 2.1-kb *Kpn*I-*Bam*HI *CaFKS1* fragment from pJAD1. The markers used are described in the legend to Fig. 2A. (B) Restriction map of homozygous *Cafks1h* locus from strain NR3. The symbols and features of the map are described in the legend to Fig. 2C. (C) Map of pAM600 integration at *Cafks1h-3* of strain NR3.

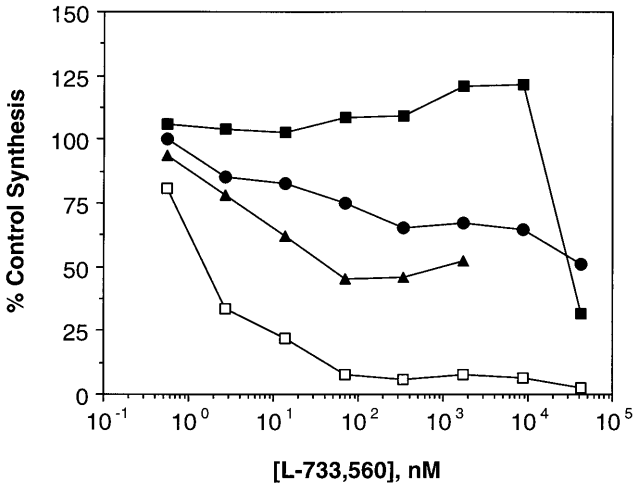

FIG. 5. Inhibition by L-733,560 of GS activity from mutant strains NR2 (●), NR3 (■), and NR4 (▲). Reactions were performed as described in the legend to Fig. 3B, except the membrane protein concentration ranged from 0.4 to 0.8 mg/ml, incubation was at 30°C, UDP-[^{3}H]glucose was at 7,500 dpm/nmol, and L-733,560 was titrated from 5.5×10^{-1} to 4.3×10^4 nM. Results with the wild-type strain (CAI4 [□]) are also shown. The specific activity of each enzyme is given in Table 3.

was pneumocandin resistant, with increases in the IC_{50}s ranging from 20-fold (NR4) to roughly 10,000-fold (NR2 and NR3). However, inhibition of GS activity from strains NR2 and NR4 reaches a maximum of ca. 55%, with a shallow slope over a wide range of L-733,560 concentrations. This profile resembles the curve for pneumocandin inhibition of CAI4R1 GS (Fig. 3B). In contrast, GS activity from strain NR3 was entirely resistant to inhibition with L-733,560 at concentrations below 10 μM. The shape of this curve is very similar to those of the titration curves shown for inhibition of enzyme activity from strains T25 and T26 (Fig. 3B).

We also evaluated the susceptibility of several transformants and their parents to antifungal antibiotics currently in clinical use (Table 2). Amphotericin B, itraconazole, fluconazole, nystatin, ketoconazole, and 5-fluorocytosine produced equivalent zones of inhibition against all nine strains. Strains T25 and T26 and the Ura$^+$ pJAM15 transformants of strains NR2, NR3, and NR4 were also resistant to several known GS inhibitors, including echinocandin B, several pneumocandins, and dihydropapulacandin (data not shown). This specificity of resistance is characteristic of *S. cerevisiae* mutants selected for resistance to GS inhibitors, such as strains R560-1C (12) and *pbr1-1* (9).

Two-step integrative transformation at *CaFKS1*. We sought to determine whether *CaFKS1* is an essential gene. Our strategy was to transform Ura$^-$ strains bearing one disrupted allele at the *CaFKS1* locus with a *URA3*-containing disruption plasmid that also targets *CaFKS1* and determine the genotypes of the Ura$^+$ transformants by Southern blot analysis. If *CaFKS1* is not required for viability, we expected to recover clones with disruptions of both copies of the gene. To disrupt the first allele, strain CAI4 was transformed to Ura$^+$ with plasmid pAM602, which had been digested with *Pvu*II and *Kpn*I to release the *Cafks1*::*hisG-URA3-hisG* insert. Clones were purified on SC Ura$^-$, and genomic DNA was analyzed on Southern blots (Fig. 6). Transformant T6 had a disruption of the *Cafks1b* allele (*CaFKS1h*/*Cafks1b*::*hisG-URA3-hisG*); digestion of genomic DNA from strain T6 with *Hin*dIII and *Pvu*II yielded 6.7- and 12-kb fragments. Several independent colonies

TABLE 2. Susceptibilities of CAI4-derived strains to antifungal antibiotics in clinical use

Compound	Amt (μg/spot)	Diam of zone of inhibition (mm) with:								
		CAI4	CAI4R1	T25	T26	T28	T32	NR2(Ura$^+$)	NR3(Ura$^+$)	NR4(Ura$^+$)
Amphotericin B	5	19	16	17	17	17	17	16	18	18
Itraconazole	10	20	18	19	20	19	18	17	18	20
Fluconazole	5	25	20	20	20	24	21	22	20	19
Nystatin	10	24	20	20	21	20	20	18	24	23
Ketoconazole	5	33	31	33	32	32	31	31	29	30
5-Fluorocytosine	10	16	14	16	16	15	14	14	16	16

were transferred to plates containing 5-fluoroorotic acid (5FOA) and uridine to select for loss of the *URA3* gene (16). The Southern blot of *Hin*dIII-*Pvu*II-digested DNA from one Ura$^-$ derivative (T6FOA1) revealed two fragments (6.7 and 9.3 kb), consistent with loss of the *URA3* gene but retention of one *hisG* insertion (*CaFKS1*/*Cafks1b*::*hisG*). The CAI4 transformant T1 had a disruption of the *CaFKS1h* allele; genomic DNA from T1 yielded 8.5- and 15-kb fragments upon digestion with *Hin*dIII and *Pvu*II (Fig. 6). The size of the insertion-bearing allele was consistent with tandem integration of two cassettes (*Cafks1h*::*hisG-URA3-hisG-URA3-hisG*/*CaFKS1b*). This structure was confirmed with an additional Southern blot; digestion of DNA from strain T1 with each of three restriction enzymes that cut once in the 3.9-kb *hisG-URA3-hisG* cassette produced a 3.9-kb fragment that hybridized with a *hisG* probe (data not shown). A Ura$^-$ derivative (T1FOA3) obtained following selection with 5FOA was also analyzed (Fig. 6), and the fragment pattern (7.5- and 8.5-kb *Hin*dIII-*Pvu*II fragments) was consistent with retention of a single *hisG* cassette (*Cafks1h*::*hisG*/*CaFKS1b*).

A second round of targeted disruption at the *Cafks1* locus was performed by transforming plasmid pAM601, linearized by digestion with *Sal*I, into strains T6FOA1 and T1FOA3. Uridine prototrophs were selected on SC Ura$^-$, and genomic DNA was analyzed on Southern blots. Among 22 transformants of strain T1FOA3, 16 yielded the pattern expected for the genotype *Cafks1h*::*URA3*/*CaFKS1b* (Fig. 6 and data not shown). The remaining transformants had more complex fragment patterns, but all had an intact 8.5-kb *CaFKS1b* allele. We obtained similar results with strain T6FOA1; among 13 transformants, none had the pattern expected for a disruption of the functional 6.7-kb *CaFKS1h* allele (Fig. 6 and data not shown). Clones were obtained by both spheroplast and lithium acetate transformation methods. Because we were able to isolate numerous *Cafks1*::*URA3*/*CaFKS1* transformants but no *Cafks1*::*hisG*/*Cafks1*::*URA3* transformants, we propose that a functional *CaFKS1* allele is essential for viability under these growth and transformation conditions.

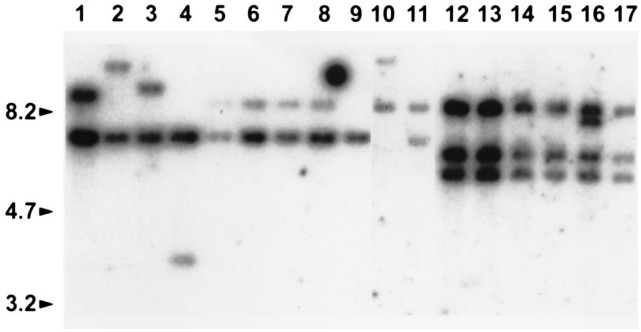

FIG. 6. Southern blot of clones from two-step integration at the *CaFKS1* locus. DNA from each strain was analyzed as described in the legend to Fig. 2. pAM602 transformation of wild-type strain CAI4 (lane 1) produced transformants with plasmid integration in either the *CaFKS1b* allele (strain T6 [lane 2]) or the *CaFKS1h* allele (strain T1 [lane 10]). Clones selected for 5FOA resistance (strain T6FOA1 [lane 3] and strain T1FOA3 [lane 11]) were transformed with pAM602, and the fragment profiles for seven Ura$^+$ transformants of strain T6FOA1 (T54 through T59 [lanes 4 to 9, respectively]) and six transformants of strain T1FOA3 (T3 through T8 [lanes 12 to 17, respectively]) are shown. The positions of markers (as described in the legend to Fig. 2, with the addition of a 3.2-kb standard from pJAD1DXH digested with *Bam*HI and *Sal*I) are indicated (in kilobases).

Growth rates, virulence, and susceptibility to L-733,560 in vivo. The reduced growth rate of *S. cerevisiae fks1* null mutants and the poor growth and weak virulence of the *C. albicans* Ech^r mutant M2 (27) confirm that changes in GS activity can be detrimental to the cell. For strain CAI4R1, it was shown that the spontaneous Ech^r mutation had no significant effect on growth in vitro or on the ability to establish a disseminated infection in immunocompromised mice (27). Animals infected with a Ura$^+$ derivative of this heterozygous mutant required an ED_{99} of L-733,560 approximately eightfold higher than that for animals infected with strain CAI4(Ura$^+$) (27). We evaluated the consequences of single-allele disruption at the *CaFKS1* locus on both growth rate and GS activity in vitro and on the LD_{50} and ED_{99} of L-733,560 in vivo (Table 3). The doubling times of all strains we tested were between 80 and 110 min in YPDAUd medium; there was no dramatic effect of the Ech^r mutations or loss of either the *CaFKS1h* or *CaFKS1b* allele on the growth rate. The specific activity of GS from these strains (Table 3) ranged from 13 to 55 nmol · mg^{-1} · h^{-1}; for each strain, the GS activity varied for replicate membrane preparations, such that differences of less than twofold should not be considered significant. Therefore, cells with only a functional *CaFKS1b* allele (T32) or *CaFKS1h* allele (T25) were not significantly impaired in their ability to grow in liquid culture or express GS activity. In an animal model of disseminated candidiasis, the LD_{50}s ranged from a low of 4,500 CFU/mouse [strain NR2(Ura$^+$)] to a high of 330,000 CFU/mouse (strain T6). Lethality relative to the functional diploid CAI4(Ura$^+$) was slightly reduced (less than twofold) when the *CaFKS1h* allele was disrupted in strain T1, but disruption of the *CaFKS1b* allele in strain T6 produced a nearly 65-fold reduction in the LD_{50}. Among the heterozygous Ech^r mutants, strain NR4(Ura$^+$), with a mutant *Cafks1b* allele (*Cafks1b-4*/*CaFKS1h*) was nearly 32-fold less virulent than strain CAI4 (Ura$^+$), while the LD_{50}s for two strains with wild-type *CaFKS1b* alleles [CAI4R1(Ura$^+$), *Cafks1h-1*/*CaFKS1b*; NR2 (Ura$^+$), *Cafks1h-2*/*CaFKS1b*] were essentially equivalent to that of CAI4(Ura$^+$). Strain NR3(Ura$^+$), which was homozygous for the *Cafks1h-3* allele, was only twofold less virulent than the wild-type diploid. Finally, the CAI4R1-derived transformants T25 and T26 (*Cafks1h-1*/*Cafks1b*::*URA3*), as well as strains T28 and T32 (*Cafks1h-1*::*URA3*/*CaFKS1b*), were all roughly 3- to 10-fold less virulent than strain CAI4(Ura$^+$).

TABLE 3. Analysis of mutant strains for growth and GS activity in vitro and virulence and pneumocandin susceptibility in vivo

Strain	Genotype	In vitro		In vivo		
		Doubling time (min)	GS sp act[a]	14-day LD_{50} (10^4 CFU/mouse)	ED_{99} (mg/kg)	
					L-733,560	AMB[c]
CAI4(Ura$^+$)	*CaFKS1h*/*CaFKS1b* (*pJAM15*)	90	31	0.51	0.06	0.05
T1	*Cafks1h*::*hisG-URA3-hisG-URA3-hisG*/*CaFKS1b*	ND[b]	ND	0.63	ND	ND
T6	*CaFKS1h*/*Cafks1b*::*hisG-URA3-hisG*	ND	ND	33	ND	ND
CAI4R1(Ura$^+$)	*Cafks1h-1*/*CaFKS1b* (*pJAM15*)	93	55	0.47	0.25	0.03
T25	*Cafks1h-1*/*Cafks1b*::*URA3*	85	24	1.7	>6.0	0.14
T26	*Cafks1h-1*/*Cafks1b*::*URA3*	90	22	5.1	>6.0	0.05
T28	*Cafks1h-1*::*URA3*/*CaFKS1b*	110	13	2.8	0.11	0.04
T32	*Cafks1h-1*::*URA3*/*CaFKS1b*	100	35	3.7	0.03	0.02
NR2(Ura$^+$)	*Cafks1h-2*/*CaFKS1b* (*pJAM15*)	ND	52	0.45	0.74	0.12
NR3(Ura$^+$)	*Cafks1h-3*/*Cafks1h-3* (*pJAM15*)	ND	53	1.1	>6.0	0.05
NR4(Ura$^+$)	*CaFKS1h*/*Cafks1b-4* (*pJAM15*)	80	19	16	0.75	0.07

[a] Expressed as nanomoles of product · $hour^{-1}$ · milligram of $protein^{-1}$.
[b] ND, not determined.
[c] AMB, amphotericin B.

We evaluated the susceptibilities of these strains to treatment with L-733,560 in a mouse model. Animals were infected with a suspension of each strain at 0.5 to 1 times the 14-day LD_{50} and treated with L-733,560 or amphotericin B according to the regimen outlined in Materials and Methods. The ED_{99}s for L-733,560 (Table 3) can be used to categorize the strains into three distinct classes: strains T28, T32, and CAI4(Ura$^+$) were very susceptible to the antibiotic; strains CAI4R1(Ura$^+$), NR2(Ura$^+$), and NR4(Ura$^+$) were less susceptible, requiring 4 to 13 times more L-733,560 for 99% efficacy; and strains T25, T26, and NR3(Ura$^+$) were not significantly eliminated from infected mice, even at the highest dose of L-733,560 we tested. The Ech^r mutations did not confer cross-resistance to amphotericin B; ED_{99}s for all strains, including the T25, T26, and NR3(Ura$^+$) mutants, were within the range of 0.02 to 0.14 mg per kg. Therefore, the four independent mutations conferred in vitro pneumocandin resistance in a liquid broth microdilution assay and a GS assay and caused an increase in the ED_{99}s for L-733,560 treatment of disseminated candidiasis in an animal model.

DISCUSSION

Numerous studies have identified the echinocandins as GS inhibitors with the potential for use as novel antifungal drugs to treat human mycoses (31). With the development of potent inhibitors of GS and the growing repertoire of genetic and molecular methods for *C. albicans*, we have the tools to study the role of this essential enzyme for cell wall synthesis. Work with the nonpathogen *S. cerevisiae* has shown that the GS subunit Fks1p is the likely target of echinocandin inhibition. The *S. cerevisiae etg1-1* strain contains a semidominant mutation that leads to echinocandin-resistant whole cells with resistant GS activity. Recent studies with the *C. albicans* strain CAI4 have identified rare spontaneous Ech^r mutants that are amenable to genetic analysis and can be evaluated for virulence. Because these mutants had phenotypes similar to those of the *S. cerevisiae etg1-1* mutant, we suspected that the mutant Ech^r allele in these isolates might be a homolog of *FKS1*. Here, we have provided evidence that the pathogen *C. albicans* has a genetic segment specifying an Fks1p homolog and that the rare mutations are indeed dominant alleles of this gene (*CaFKS1*). Our inability to isolate homozygous null mutants in *CaFKS1* suggests that it is essential for survival of *C. albicans.*

The *CaFKS1* gene fragment we cloned from *C. albicans* CAI4 has considerable homology to *FKS1*. The amino acid sequence predicted from this fragment is 689 residues long, with ca. 75% identity to the Fksp family of proteins (Fks1p, Fks2p, and FksAp). The identity between these proteins is higher (88%) if the comparison is limited to the region of each protein within the large, predicted cytoplasmic domain. While this fragment of *CaFKS1* was sufficient for our analysis, it is not the entire open reading frame. It remains to be seen if other domains of CaFks1p have significant identity to the equivalent regions of the proteins from *A. nidulans* and *S. cerevisiae*. Preliminary data from cloning and sequencing of an *FKS* homolog from *C. neoformans* suggests that this gene product is also remarkably conserved (23). Unlike genes of the *FKS* family, genes encoding proteins involved in chitin biosynthesis (*CHS* genes) are significantly divergent across different fungal genera (7), and three isozymes from *C. albicans* (CaChs1p, CaChs2p, and CaChs3p) are significantly different from one another (7, 19, 38, 44, 45).

Integration of the *URA3*-based disruption plasmid pAM600 into the *Cafks1* locus of three spontaneous *C. albicans* Ech^r mutants yielded a mixture of Ech^s and Ech^r transformants. Loss of the resistance phenotype in Ech^s transformants was correlated with disruption of the *Cafks1h* allele for strains CAI4R1 and NR2 and with disruption of the *Cafks1b* allele for strain NR4. The results with strain CAI4R1 confirm a previous study based on spheroplast fusions with genetically marked Ech^s strains (27), in which the Ech^r phenotype was found to be semidominant. Even though the diploid strain CAI4R1 (*Cafks1h-1*/*CaFKS1b*) is consistently more susceptible in vitro to L-733,560 than strains lacking a functional Ech^s allele [T25 and T26 (*Cafks1h-1*/*Cafks1b*::*URA3*)], the difference is small (Fig. 3). Because we recovered both resistant and sensitive pAM600 transformants of CAI4R1, NR2, and NR4, the *Cafks1h-1*, *Cafks1h-3*, and *Cafks1b-4* mutations must be considered dominant. In addition, both alleles must be expressed under the growth conditions used in our study, since a mutation in either the *CaFKS1h* allele or the *CaFKS1b* allele permits Ech^r/Ech^s heterozygotes to grow on medium supplemented with L-733,560 sufficient to kill Ech^s/Ech^s cells.

Strain NR3 was unique among the echinocandin-resistant mutants we isolated. First, only one allele was detected on Southern blots of NR3 genomic DNA digested with *Hin*dIII and *Pvu*II, and the size was consistent with that of the *CaFKS1h* allele. Second, pAM600 transformants of strain NR3

were uniformly Ech^r; we recovered no Ech^s derivatives among the 75 clones we evaluated. The fragment pattern observed on Southern blots of three transformants suggested that these strains contained both an intact and a disrupted copy of the *CaFKS1h* allele (Fig. 4). While we cannot rule out the possibility of a dominant Ech^r mutation at another locus, it seems likely that strain NR3 has two resistant alleles at *CaFKS1*. Under selective pressure imposed by L-733,560, an original mutant allele could have been duplicated by gene conversion. Such a mutation at the *CaFKS1* locus could be either recessive or dominant. If the mutation were recessive, it would support the idea that two types of mutations in *FKS* genes can confer resistance to glucan synthase inhibitors in the laboratory, i.e., recessive or very weakly dominant mutations, such as the *S. cerevisiae etg1-4* (15) and *pbr1-1* (9) mutations, as well as dominant mutations typified by the *Cafks1h-1* mutation.

Our Southern blots revealed a fortuitous restriction fragment length polymorphism at the *CaFKS1* locus that was instrumental for distinguishing the two alleles and demonstrating that disruption of either allele was not overtly deleterious under laboratory growth conditions, but a second forced integration yielded only transformants with the plasmid integrated into the preexisting disrupted allele. These results suggest that loss of both copies of the *CaFKS1* gene was either a lethal event or sufficiently disabling that viable transformants could not be detected. This observation is consistent with our inability to detect a homolog of *CaFKS1* under stringent conditions, like the *FKS2* gene of *S. cerevisiae* which can rescue cells lacking a functional *FKS1* gene. For *A. nidulans* (25), only one *FKS* homolog was detected by restriction mapping and hybridization analysis. A 0.4-kb *C. albicans* gene fragment with 47% homology to the *S. cerevisiae FKS2* gene was identified by random sequence analysis of the *C. albicans* genome (42), but the role of this gene (*CaFKS2*) is not understood.

The disruption strategy we used has been employed by others to identify essential genes in *C. albicans*, including *HSP90*, a member of the heat shock protein 90 family of molecular chaperones (46), and *KRE6*, a gene required for β-1-6 glucan synthesis (39). Besides *KRE6*, several other important but nonessential genes for *C. albicans* cell wall synthesis and structure have been identified. The *CHS2* (19) and *CHS3* (44) genes play a role in chitin synthesis; there is evidence that *CHS3* plays an important role in virulence in animal models of infection (8). A third gene (*CHS1*) has been identified, but its role remains unclear (38, 45). Mutants in the *C. albicans PHR1* gene are unable to conduct apical growth of either the yeast or hyphal growth form at alkaline pH. The Phr1 protein is produced at more alkaline pHs and shows strong identity to the Gas1p glycosylphosphatidylinositol-anchored cell wall protein from *S. cerevisiae*. It is interesting that *GAS1* is not an essential gene in *S. cerevisiae*, but loss of *GAS1* combined with disruption of either *KRE6* or *PKC1* is synthetically lethal (40).

Virulence and in vivo susceptibility to treatment with L-733,560 were assessed for several strains in a mouse model of disseminated candidiasis. Although each strain we tested was able to establish a lethal infection, the LD_{50}s were not equivalent, ranging from 0.45×10^4 to 33×10^4 CFU/g of kidney, and neither growth rate nor GS specific activity was predictive of the relative virulence. Reduced virulence also does not seem to be associated with disruption or mutation of a specific allele. While the wild-type *CaFKS1b* allele is preserved in several strains that have low LD_{50}s [CAI4(Ura$^+$), T1, CAI4R1(Ura$^+$), and NR2(Ura$^+$)], a strain that lacks the *CaFKS1b* allele entirely [NR3(Ura$^+$)] and two transformants with a disrupted *Cafks1b*::URA3 allele (strains T25 and T26) still showed significant virulence in the mouse model (Table 3). It is possible that other mutations, either spontaneous or induced during transformation, are responsible for the differences in virulence. We also were unable to predict from liquid broth microdilution assays or GS IC_{50}s which strains would be most resistant in vivo to treatment with pneumocandin. Strain CAI4R1(Ura$^+$) resembles strains T25 and T26 in its susceptibility in vitro to L-733,560 (Fig. 3A), but the in vivo susceptibilities were not equivalent; infections with strains T25 and T26 required at least 24-fold more L-733,560 for 99% effective treatment than infections with strain CAI4R1(Ura$^+$) (Table 3). Likewise, the IC_{50}s of L-733,560 for GS activities from strains NR2, NR3, and NR4 (40,000, 30,000, and 40 nM, respectively [Table 2]) predict that strains NR2(Ura$^+$) and NR3(Ura$^+$) should be equally resistant in vivo and that strain NR4(Ura$^+$) should be significantly more susceptible. However, in infected animals, strain NR3(Ura$^+$) was the most resistant to L-733,560 ($ED_{99} > 6.0$), while ED_{99}s for strains NR2(Ura$^+$) and NR4(Ura$^+$) were equivalent (0.74 and 0.75, respectively), with values at least eightfold lower than that for strain NR3(Ura$^+$). A qualitative measure of enzyme resistance, the shape of the GS titration curves for L-733,560 inhibition, shown in Fig. 3B and 5, represents the best prediction of pneumocandin susceptibility in vivo. The Ech^r/Ech^s heterozygous mutants are of intermediate resistance in vivo, and their GS inhibition curves suggest intermediate resistance, with a shallow slope and a maximum of 55% inhibition. We do not know what extent of GS inhibition is sufficient to clear a cell from an infected animal, so the use of GS IC_{50}s as we have defined them may not be warranted to predict phenotypes in vivo. In addition, variables of the in vitro GS assay such as the concentration of crude membrane protein and the incubation temperature can have a significant effect on the absolute IC_{50}, as evidenced by the results for GS from strain CAI4 shown in Fig. 3B and 5.

The Ech^r mutants we have characterized in this paper were isolated at high cell densities on agar plates containing the pneumocandin L-733,560. Although these strains represent a useful tool for genetic characterization, their relevance to the potential for clinical pneumocandin resistance is unclear. Ech^r organisms were not recovered in a *C. albicans* target-organ model of infection when pneumocandin MK0991 (formerly L-743,872) was given to neutropenic mice via intraperitoneal administration (1, 43). The outcome of current and future use of echinocandins and pneumocandins in humans will provide relevant information about the potential for clinical resistance to this new class of antifungal antibiotics.

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