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Coccidioides posadasii Contains a Single 1,3-β-Glucan Synthase Gene That Appears To Be Essential for Growth

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1,3-β-Glucan synthase is responsible for the synthesis of β-glucan, an essential cell wall structural component in most fungi. We sought to determine whether Coccidioides posadasii possesses genes homologous to known fungal FKS genes that encode the catalytic subunit of 1,3-β-glucan synthase. A single gene, designated FKS1, was identified, and examination of its predicted protein product showed a high degree of conservation with Fks proteins from other filamentous fungi. FKS1 is expressed at similar levels in mycelia and early spherulating cultures, and expression decreases as the spherules mature. We used Agrobacterium-mediated transformation to create strains that harbor ΔFKS1::hygB, a null allele of FKS1, and hypothesize that Fks1p function is essential, due to our inability to purify this allele away from a complementing wild-type allele in a heterokaryotic strain. The heterokaryon appears normal with respect to growth rate and arthroconidium production; however, microscopic examination of strains with ΔFKS1::hygB alleles revealed abnormal swelling of hyphal elements.

Coccidioides posadasii is found only in the area of the San Joaquin Valley of California (13). Although the species are genetically distinct, differences in morphology or pathogenicity have not yet been delineated.

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Coccidioides species (C. immittis and C. posadasii) are primary fungal pathogens that cause disease by the respiratory route in humans and many other species of mammals. The genus has recently been reorganized from one species (C. immittis) into two species, with C. posadasii being localized to Texas, Arizona, Mexico, and South America whereas C. immittis is found only in the area of the San Joaquin Valley of California (13). Although the species are genetically distinct, differences in morphology or pathogenicity have not yet been delineated.

Coccidioides spp. are dimorphic and produce characteristic structures for the saprobic and parasitic growth phases (for a review of the life cycle, see reference 6). The soil-dwelling saprobic phase is mycelial, with asexual spores produced through septation of hyphae and subsequent differentiation of alternate cellular compartments into arthroconidia, which contain on average two nuclei but sometimes contain more (6). Mature conidia 3 by 6 to 2 μm in size are liberated by physical disruption and disperse readily in the wind. Upon inhalation into the host lung, the arthroconidium undergoes a phase transition to form a large (60 to >100 μm) spherule that undergoes internal septation to produce hundreds of endospores. Most infections are relatively mild and resolve without specific treatment, although about 30% of infections produce flu-like symptoms (39). However, some infections are severe and require extensive antifungal therapy (40).

The fungal cell wall is conceptually attractive as a target for antifungal therapies. It is critical for maintaining cell shape and integrity and contains the polysaccharides 1,3-β-glucan and chitin (4, 38), which are not constituents of mammalian cells. Antifungal agents that act by inhibiting their synthesis could be highly selective.

1,3-β-glucan is the predominant polysaccharide in most fungal cell walls, including those of Saccharomyces cerevisiae, Candida albicans, and Neurospora crassa (14, 38), and is a significant component in the walls of C. posadasii in all growth phases (21, 42). The glucan synthase complex has been best studied in S. cerevisiae and consists of a catalytic subunit encoded by either of two genes (FKS1 or FKS2) and a regulatory subunit (RHO1). The FKS genes encode large polypeptides (~200 kDa) with 16 membrane-spanning domains that synthesize 1,3-β-glucan from UDP-glucose subunits (4, 11, 33). Rho1p is a small GTPase that in its prenylated form interacts with Fks catalytic subunits to activate 1,3-β-glucan synthase (22, 23, 32, 37). Glucan synthase activity is essential in yeast. An FKS1 FKS2 double deletion is lethal, although single deletions are viable, suggesting overlapping functions of the two catalytic polypeptides (24, 33). FKS2 is required for sporulation, and a third gene in yeast, FKS3, is nonessential for growth or sporulation (41). FKS1 homologs have been reported in the pathogenic fungi C. albicans, Cryptococcus neoformans, Paracoccidioides brasiliensis, and Pneumocystis carinii and the filamentous fungi Aspergillus nidulans and Aspergillus fumigatus (3, 10, 28, 35, 41). The FKS1 genes have been demonstrated to be essential single-copy genes in C. albicans, C. neoformans, and A. fumigatus (3, 10, 41). The echinocandins and pneumocandins are a class of antifungal drugs that inhibit 1,3-β-glucan synthase (9). Use of one echinocandin, caspofungin (Merk Pharmaceuticals), is successful in the treatment of infections due to Candida spp. and Aspergillus spp. and has been approved by the Food and Drug Administration for such treatment. However, it has not been effective against C. neoformans (2, 25, 29, 36). Caspofungin has been shown to be effective in treating mice infected with Coccidioides spp. (19), but relatively high
doses were required, and in earlier studies a different echino-
candin, cilofungin, was found not to be useful (17). Although these results suggested that 1,3-β-glucan synthase might not be a therapeutically important antifungal target in *Coccidioides* spp., other pharmacologic factors could be responsible for these marginal effects. To investigate 1,3-β-glucan synthase as an antifungal target in *Coccidioides* spp., we sought to identify the gene and disrupt the function of the FKS homolog(s) in *C. posadasii*. We have identified a single gene, *FKSI*, with similarity to other fungal FKS genes, and disruption experiments suggest that *FKSI* is essential for growth of *C. posadasii*.

**MATERIALS AND METHODS**

Strains, media, and growth conditions. *C. posadasii* strain Silveira (ATCC 28868), originally isolated from a patient (15), was maintained in the saprobic phase on 2% GYE agar (2% glucose, 1% yeast extract, 1.6% agar) at 28°C or ambient room temperature. To isolate arthroconidia, strains were grown for 3 to 4 weeks on 2% GYE agar and arthroconidia were harvested in sterile water by use of a sterile Pasteur pipette. Spheres were washed at 39°C and 9% CO₂ in modified Converse medium (7). All manipulations of viable preparations were performed using biosafety level 3 conditions and practices in laboratories registered with the Centers for Disease Control for possession of this select agent.

**Isolation and sequencing of FKS1.** A *C. posadasii* genomic library constructed in lambda GEM13 (34) was screened with a 405-bp probe generated by reverse transcriptase PCR (RT-PCR) from *mycelial* RNA by use of degenerate primers based on the *A. nidulans* FKSAp (26), asp4U ([5’GCAAAACARGAYAAYTA] and asp5L ([5’YTCRAGTYTTT]) the probe was tagged with digoxi-
genin-UTP (DIG-UTP) according to the vendor’s protocol by use of a Roche “Genius” kit (Boehringer Mannheim, Indianapolis, Ind.). The hybridizing λ clone GS5 was digested with SacI, and two subfragments of 3.5 and 6.5 kb were cloned into pBluescript SK− (Stratagene, La Jolla, Calif.) to create pES518 and pES527, respectively. The insert of pES518 was sequenced in its entirety and found to contain sequence with similarity to fungal FKS gene sequences encoding 1,3-β-glucan synthase catalytic subunits. The predicted start and end of the FKS open reading frame were not present in this subclone. The carboxy-terminal sequence of the predicted glucan synthase gene as well as downstream sequence were obtained from pES527. The sequence of a 500-bp SacI fragment predicted into pBluescript SK− (Stratagene, La Jolla, Calif.) to create pES518 and pES527 was obtained from direct sequencing of the λ clone GS5. A partial sequence was deposited in GenBank (GenBank AF195533).

To obtain further upstream sequence, including the predicted start of the open reading frame, additional λ clones were screened. A 508-bp PCR fragment was generated from the 5′ proximal sequences of pES518 by use of primers ESGS12 ([5’TATAGACCGTGTTCTC] and ESGS9 ([5’TATCGCTGTTCTACGTA] the PCR product was labeled with [γ-32P]dCTP and used to probe Southern blots containing EcoRI digests of four additional λ clones isolated during the original screening. A 3-kb hybridizing fragment of one clone, GS1, was cloned into pBluescript SK+ (Strategene), generating pAM1203. The insert of pAM1203 was sequenced on both strands and found to contain the predicted N-terminal regions of the *C. posadasii* 1,3-β-glucan synthase homolog designated FKS1.

Construction of pAM1253 with a FKS1 deletion-replacement cassette. The plasmid pAM1253 that contained the FKS1 gene replacement cassette was created in multiple steps. A 1-kb PCR fragment was generated from the 5′ FKS1-proximal sequence in pAM1253 by use of primers OAM472 ([5’CCCCAGTTC CCAATTTAGCCCGAGG ATC] and OAM473 ([5’CCGGTACCAT CATGTAATCCAGG AAG]), OAM472 and OAM473 were designed to add 5′ EcoRI and 3′ KpnI restriction sites to the PCR product. The PCR fragment was first cloned into pGEM-T Easy (Promega) to create pAM1245. A 1-kb EcoRI-KpnI fragment was isolated from pAM1245 and cloned into the EcoRI and KpnI sites of pAM1211, creating pAM1248 and placing the FKS1 upstream sequence 5′ to the hphB gene cassette derived from pCB1004 (5). A 2-kb PCR fragment was generated from sequence 3′ of the FKS1 gene by use of primers OAM482 ([5’GGATCCTTGCCTTGTGGTC ] and OAM483 ([5’GAATC ATCTGTTGTTCTACGCACTAC]), and genomic DNA as a template. The primers were designed to add 5′ BamHI and 3′ BglII restriction sites to the PCR product. The PCR fragment was cloned into pGEM-T Easy to create pAM1247. To complete construction of the FKS1 gene replacement vector, fragments containing the 3′ flanking sequence, the 5′ flanking sequence fused to hphB, and the binary vector pAM1145 were ligated together. The 3′ flanking sequence were released from pAM1247 as a 2-kb BamHI-BglII fragment; the 5′ flanking sequence and hphB were released from pAM1148 as a 2.6-kb EcoRI-BamHI fragment, and these were cloned into pAM1145 digested with EcoRI and BglII to create pAM1253. The binary vector pAM1145 contains a broad-host- range origin of replication, the right and left borders of the T-DNA from the Escherichia coli plasmid pTiA6, and a mutated form of virG (virG<sup>2052</sup>), which obviates the need to add compounds such as acetosyringone to induce T-DNA transfer (26). pAM1145 was previously constructed by insertion of a 5.6-kb KpnI-SalI fragment from plasmid pDAD1378 (8) into pAD1310 (27). pAM1253 was introduced into *A. tumefaciens* strain A9D65 by electroporation to create strain A1253.

**Transformation of C. posadasii.** *C. posadasii* was transformed using *A. tumefaciens* strain A1253 as described previously (1), with some modifications as follows. Arthroconidial gernings and *A. tumefaciens* were mixed and dispensed with a sterile spatula onto sterile 80-mm-diameter nitrocellulose filters placed onto 100-mm-diameter petri plates (seven plates) containing AB induction medium. Plates were incubated at 28°C, and after 48 h the nitrocellulose filters were transferred to plates containing 2% GYE agar with 50 μg of hygromycin (Hyg/ml) to select for transformed *C. posadasii* and 100 μg of kanamycin/ml as a counterselection for *A. tumefaciens*. Transformants were cut from the nitrocellulose filters and transferred to fresh plates of the selection medium, and allowed to conidiate. This conidial passage was repeated two more times.

**Southern blot analysis of transformants.** *C. posadasii* genomic DNA was isolated from about 0.1 g wet weight) from a young colony, transferring it to a 2-ml screw-cap tube containing about 600 μl of 0.5-mm-diameter acid-treated glass beads and 1 ml of lysis buffer (50 mM Tris-HCl [pH 7.5], 100 mM EDTA [pH 8.0], 100 mM NaCl, 0.5% sodium dodecyl sulfate, 100 mM dithiothreitol), and subjecting it to mechanical disruption. The tubes were shaken either by bead beating (Mini Beadbeater 8; Biospec Products, Bartlesville, Okla.) for three periods of 30 s with 20-s rest intervals in between or by vortexing them at a flat 12 rpm for 100 rpm for 10 min, incubated at 5°C for 30 min and centrifuged in a microcentrifuge at 9,000 × g for 2 min. The supernatant was extracted with phenol-chloroform-isooamyl alcohol (25:24:1), and nucleic acids were precipitated from the aqueous layer with 0.6 volumes of isopropanol alcohol. Pellets were resuspended in 300 μl of Tris-EDTA (pH 7.6) containing 100 μg of RNAse A/ml and incubated at 37°C for 30 min. A total of 50 μl of 5 μM NaCl and 40 μl of cyctylintrimyllumin monium bromide-NaCl (10% cyctylintrimyllumin monium bromide (0.7 μM NaCl) was added to each sample, and the samples were incubated at 68°C for 10 min. The samples were extracted twice with equal volumes of chloroform-isooamyl alcohol, once with phenol-chloroform-isooamyl alcohol, and once again with chloroform-isoamyl alcohol. The DNA was precipitated with isopropanol alcohol and resus-

**RNA isolation and real-time RT-PCR analysis.** RNA was prepared from my-
celia (48 h of growth in liquid 2% GYE agar at 37°C) and spheres (grown in modified Converse liquid medium at 37°C and 8% CO₂). Mycelia were harvested by filtration onto sterile Whatman Type 1 filter paper and washed with ice-cold RNase-

**Poly(A)⁺ RNA was isolated from 300 μg of total RNA from each develop-
mental time point by use of a PolyATrac mRNA isolation system III (Promega, Madison, Wis.) following the manufacturer’s recommended protocol. RNA concentration were estimated using RiboGreen RNA quantitation reagent (Molecular Probes, Inc., Eugene, Oreg.) and an FLX 800 fluorometer (Bio-Tek Instruments, Inc., Winooski, Vt.). Poly(A)⁺ RNA (7.5 mg) was used to synthesize first-strand cDNAs from RNA isolated from mycelia and 48-h, 72-h, and 96-h
spheres by use of oligo(dT) 12 to 18 and SuperScript Reverse Transcriptase II (Invitrogen, Sorrento Valley, Calif.) following the manufacturer’s protocol. Following treatment with RNase H (Invitrogen), specific oligonucleotide primers for \( \text{FKS1} \) and for \( \beta \)-actin were used in real-time RT-PCR experiments with SYBR Green PCR Master Mix in an ABI PRISM 7000 sequence detection system (Applied Biosystems). The oligonucleotides used for \( \text{FKS1} \) were OAM697 (GC ATTTCGATCTCCTCAACCCG) and OAM698 (CGGAAATGGAATCGGCGAC CG); those used for \( \beta \)-actin were OAM637 (AGCTCTTGCGGTCGGAAA) and OAM638 (CAGGCATGACGATGGTTTC). Serial twofold dilutions of the first-strand cDNA were created, and a 0.125-fold dilution was used for the real-time RT-PCR experiments described here. The real-time RT-PCR experiments were performed in triplicate for each dilution, and mean cycle thresholds (Ct) were determined. A biological replicate was also performed using RNA isolated from \( C. \) posadasii cultures grown a second time.

Relative quantification calculations were performed using the algorithm \( 2^{-\Delta \Delta C_{t}} = \text{SPE} \), where \( \Delta C_{t} \) is the factor by which the amount of each gene at a certain time point in spherule development has changed relative to the expression of the same gene in mycelia (31). \( \Delta \Delta C_{t} = (C_{\text{FKS1}} - C_{\text{CtBam}})_{\text{mycelium}} - (C_{\text{FKS1}} - C_{\text{CtBam}})_{\text{mycelium}} \), where “time point” refers to spheres of 48, 72, or 96 h. \( \beta \)-actin was used as an internal control for RNA yield. SPE is the standard propagation error determined as plus or minus the square root of \( (\sigma_{\text{CtFKS1}})^{2} + (\sigma_{\text{CtBam}})^{2} \), with \( \sigma \) being the standard deviation error. Real-time RT-PCR data for \( \text{FKS1} \) expression at all time points were normalized to the expression of \( \beta \)-actin for that time point, and the normalized expression of \( \text{FKS1} \) during spherule development time points was normalized to \( \text{FKS1} \) expression in mycelia.

**Measurement of growth rates and \( \Delta \text{FKS1:phb} \) stability.** Colossal radial growth rates were measured through point inoculation of purified arthroconidial suspensions onto the centers of \( Y \) agar plates in both the presence and absence of 50 \( \mu \)g of hygromycin/ml. Each transformant strain was inoculated onto three replicates of \( Y \) agar with no antibiotic and three replicates of \( Y \) agar plus hygromycin. The culture plates were incubated at 28°C, and colony diameters were measured at 24- and 48-h intervals. Measurements used to calculate radial growth rates were those taken after 5, 6, and 8 days of growth during the linear phase of colony expansion—after a colony of sufficient size was formed but before it had covered the surface of the petri plate. After 4 weeks of incubation when the plates were entirely covered by fungal material, arthroconidia were harvested from two of the \( Y \) agar replicates and two of the \( Y \) agar plus-hygromycin replicates per strain by use of the mini-spin bar technique. Total CPU and hygromycin-resistant CPU levels were determined by plating dilutions of the same conidium preparation onto \( Y \) agar and \( Y \) agar plus plus 50 \( \mu \)g of hygromycin/ml. Yields are expressed as CFU levels (in square millimeters) over the area of the plate from which the conidia were harvested.

**Microscopy.** Sterile coverslips were placed in petri plates, and a very thin layer of 2\( \times \) \( Y \) agar (plus 50 \( \mu \)g of hygromycin/ml for transformant strains) was poured over the coverslips. A liquid suspension of arthroconidia at 5 \( \times \) 10\textsuperscript{6}/ml in \( Y \) agar (plus 50 \( \mu \)g of hygromycin/ml for transformant strains) was prepared, and a thin layer of this suspension was poured onto the agar plates with embedded coverslips. Caspofungin was used in some experiments at a concentration of 800 ng/ml in PBS. An internal staining solution (calcofluor; Sigma) was added to the petri plates to cover the surface and the plates were incubated at room temperature for 45 min. The fixation solution was removed and the fixed material was washed briefly by overlaying with distilled water. Staining solution (calcofluor; Sigma catalog no. F-3543) (10 \( \mu \)g/ml) and DAPI (diamino-2-phenylindole dihydrochloride) (Sigma catalog no. D-9542) (800 ng/ml) in PBS was then added to the plates, and the staining was allowed to proceed for 30 min at room temperature. The fixed and stained material was washed briefly with distilled water followed by 100% ethanol. Coverslips with thin agar layers were excised from the plates and mounted on microscope slides in mounting medium (Vectashield Hard Set [catalog no. H1400}; Vector Laboratories, Burlingame, Calif.). Slides were visualized using a Diaplan compound microscope (Leitz, Wetzlar, Germany) with a mercury-argon lamp and a Chroma standard filter set for DAPI (excitation D360, emission D460). Images were acquired using an Orca 100 digital camera (Hamamatsu Corporation) and Simple PCI image analysis software (Compix, Inc.).

**RESULTS**

Identification and sequencing of a \( C. \) posadasii \( \text{FKS} \) homolog. \( C. \) posadasii genomic library was screened for clones with homology to fungal \( \text{FKS} \) genes. Several \( \lambda \) clones were isolated and were found to contain sequences that encode a predicted protein with a high degree of similarity, by BLASTX analyses, to 1,3-\( \beta \)-glucan synthase (\( \text{FKS} \)) genes examined in other fungal species (3, 10, 11, 26, 35, 41). The 5,821-nucleotide sequence from \( C. \) posadasii was designated \( \text{FKS1} \) and has been deposited in GenBank (update to accession number AF159533). Analysis of \( \text{FKS1} \) sequence showed two predicted introns at coding nucleotide positions 175 to 234 and 5489 to 5549 (with the ATG start codon = position 1) with conserved donor and acceptor splice sites for filamentous fungi. Sequencing of cDNAs obtained by RT-PCR confirmed these intron-exon splice sites. The intron positions in \( \text{FKS1} \) are conserved with respect to the sequences of \( \text{FKS} \) homologs present in the related filamentous fungi \( P. \) brasiliensis (\( \text{FKS1} \); AF148715) and \( A. \) nidulans (\( \text{FKS4} \); ENU51272). The \( \text{FKS1} \) sequence was used in a BLASTN search of the genome sequence of \( C. \) posadasii strain \( C735 \) at The Institute for Genomic Research (TIGR, Bethesda, Md.), which is presently at \( 8 \times \) coverage. A single contig, containing sequence identical to that of \( \text{FKS1} \) within the coding region, was identified and was predicted to encode a peptide product identical to Fks1p. In addition, a TBLASTN search of the same genomic sequence failed to identify other translated open reading frames with similarity to the Fks1p polypeptide. These findings, combined with Southern analyses using a probe generated from \( \text{FKS1} \) internal sequences (data not shown), predict that \( C. \) posadasii contains a single gene, \( \text{FKS1} \), encoding a 1,3-\( \beta \)-glucan synthase catalytic subunit.

The predicted Fks1p protein is 1,902 amino acids and has a predicted molecular mass of 217 kDa and a pI of 8.72. The proteins that contain the highest degree of similarity to the predicted Fks1p protein of \( C. \) posadasii are Fks1p of \( P. \) brasiliensis (84% identity) and Fks1p of \( A. \) fumigatus (81% identity). Much higher degrees of identity were found in the large hydrophilic domain of approximately 577 amino acids (Fig. 1) that is hypothesized to contain the catalytic site: \( C. \) posadasii Fks1p and \( P. \) brasiliensis Fks1p (96% identity) and \( C. \) posadasii Fks1p and \( A. \) fumigatus Fks1p (93% identity). Fks1p has also putative UDP-glucose binding consensus sequence RXTG (Fks1p; RITG, amino acids 1569 to 1572). Curiously, there is a stretch of 12 glycine residues in the N-terminal portion of the polypeptide (amino acids 9 to 20) that does not appear in any other fungal glucan synthase proteins characterized to date. It is present in both the genomic and cDNA sequences of strain Silveira that were generated and analyzed for this project and in the \( C735 \) genomic sequence (TIGR).

Real-time RT-PCR was used to examine the \( \text{FKS1} \) message levels in both liquid-grown mycelial and spherulating cultures of wild-type \( C. \) posadasii strain Silveira (Fig. 2). \( \text{FKS1} \) was found to be expressed at all time points tested. The levels of \( \text{FKS1} \) expression in two separate experiments (biological replicates) relative to the levels in the mycelial RNA are shown in Fig. 2. The results of both experiments taken together indicate that \( \text{FKS1} \) expression at 48 and 72 h of spherule formation is similar to or slightly less than its levels in mycelia and that its levels decrease as spherules mature at 96 h (Fig. 2).

**Transformation of \( C. \) posadasii with a gene replacement cassette.** To determine whether 1,3-\( \beta \)-glucan synthase activity is essential for viability of \( C. \) posadasii, as it is for other fungal species, we sought to create a strain lacking \( \text{FKS1} \). \( C. \) posadasii
germlings were transformed by *A. tumefaciens*-mediated transformation by use of a strain containing a gene replacement construct composed of the *Escherichia coli* *hphB* gene flanked by 1 kb of *FKS1* 5′ sequence upstream and 2 kb of *FKS1* 3′ sequence downstream. This construct was cloned between the T-DNA border sequences of the broad-host-range plasmid pAM1145 (Fig. 3). A total of 24 hygromycin-resistant transformants were isolated. In an attempt to obtain homokaryons, arthroconidia were streaked on agar plates and individual colonies were subcultured three times. Multiple passages were performed due to the multinucleate nature of arthroconidia, which usually contain two nuclei and occasionally contain more (6). In our experience, three passages are sufficient to purify a homokaryotic strain. A combination of PCR and Southern blot analyses was used to examine the nature of the insertions present. Six of the 24 transformants had patterns consistent with the presence of a *FKS1* replacement event, but they also contained a wild-type allele of *FKS1*. The remaining 18 transformants appeared to have resulted from a nonhomologous insertion event, and two of these strains, VFC10 and VFC13, were chosen as control strains for further analysis. Representative Southern analyses are shown in Fig. 4A for selected transformants. Southern blots containing NdeI-digested genomic DNA from the transformants VFC10, VFC12, VFC13, VFC15, VFC16, and VFC43 as well as the wild-type strain Silveira are shown in Fig. 4A. When hybridized with a fragment from the 5′/H11032 upstream sequence of *FKS1*, strains VFC12, VFC15, VFC16, and VFC43 each contained a 3.7-kb NdeI fragment indicative of a homologous gene replacement event and a 10.8-kb NdeI fragment from the wild-type *FKS1* allele (Fig. 4A). These results were consistent with the idea that these transformants were heterokaryotic, containing both the Δ*FKS1::hphB* null allele and the *FKS1*WT allele in separate nuclei. Digests of VFC10 and VFC13 exhibited a hybridizing 10.8-kb NdeI fragment and additional hybridizing fragments of approximately 2.9 and 4.7 kb, respectively. These patterns were predicted to arise from a *FKS1*WT allele and an ectopic inte-

FIG. 1. The *C. posadasii* Fks1 protein sequence is similar to Fks protein sequences from related pathogenic fungi. A graphic representation of *C. posadasii* Fks1p is shown. Features indicated are the glycine repeat region, the catalytic core domain, and the UDP-glucose consensus binding site (RITG). A multiple sequence alignment of selected fungal Fks catalytic domains is shown in the bottom half of the figure (26). The alignment was generated using ClustalW (version 1.4) and the BLOSUM (blocks substitution matrix) similarity matrix with an open gap penalty of 10, an extended gap penalty of 0.05, and a delay divergent of 40%. Shaded residues indicate amino acid identities. The predicted protein sequences of *P. brasiliensis* Fks1p (amino acids 754 to 1331) (accession number AF148715) and *A. fumigatus* Fks1p (amino acids 755 to 1332) (accession number U79728) are aligned with the *C. posadasii* Fks1p predicted protein sequence (amino acids 760 to 1337).
The digest of strain Silveira DNA showed only the single predicted 10.8-kb NdeI fragment of the wild-type FKS1 locus. The blots were stripped and reprobed with a radioactively labeled 1.4-kb hphB gene (Fig. 4B). The probe detected a 2.7-kb NdeI fragment internal to the gene replacement cassette in all six transformants but not in the wild-type Silveira strain. The 3.7-kb NdeI fragment predicted to arise from strains with a homologous gene replacement hybridized with the hphB probe only in strains VFC12, VFC15, VFC16, and VFC43. The hphB probe hybridized with the same 2.9- and 4.7-fragments as the FKS1-upstream probe in lanes containing digests from VFC10 and VFC13, respectively. No hphB-hybridizing bands were present in the lane containing the NdeI-digested genomic DNA from wild-type strain Silveira. These data support the idea that these strains arose from a single integration event, due to the presence of only one band in addition to that of the wild-type fragment. The digest of strain Silveira DNA showed only the single predicted 10.8-kb NdeI fragment of the wild-type FKS1 locus. The blots were stripped and reprobed with a radioactively labeled 1.4-kb hphB gene (Fig. 4B). The probe detected a 2.7-kb NdeI fragment internal to the gene replacement cassette in all six transformants but not in the wild-type Silveira strain. The 3.7-kb NdeI fragment predicted to arise from strains with a homologous gene replacement hybridized with the hphB probe only in strains VFC12, VFC15, VFC16, and VFC43. The hphB probe hybridized with the same 2.9- and 4.7-fragments as the FKS1-upstream probe in lanes containing digests from VFC10 and VFC13, respectively. No hphB-hybridizing bands were present in the lane containing the NdeI-digested genomic DNA from wild-type strain Silveira. These data support the idea that these strains arose from a single integration event, due to the presence of only one band in addition to that of the wild-type fragment.

FIG. 2. FKS1 RNA levels decrease during spherule maturation relative to the level in mycelia. FKS1 RNA levels were determined using real-time RT-PCR, normalized to actin as an internal control, and then expressed as severalfold changes during spherule development relative to the levels found in mycelia. The two differently shaded columns for each time point represent biological replicates.

![FKS1 Gene Structure and Deletion Plasmid](image)

**FIG. 3.** FKS1 gene structure and deletion plasmid. (A) The genomic structure of the FKS1 locus is depicted, with a scale bar below in 1-kb increments. Primers used to generate PCR fragments containing the upstream and downstream flanking sequences with added restriction sites for cloning are indicated. (B) pAM1253 is the broad-host-range plasmid used to transform *C. posadasii* and create the FKS1 deletion.
FIG. 4. Southern analysis of *C. posadasii* pAM1253 transformants. (A) Genomic DNA (10 µg) from transformant strains VFC10, VFC12, VFC13, VFC15, VFC16, and VFC43 and wild-type strain Silveira was digested with NdeI and separated on a 0.8% agarose gel. The gel was transferred to nitrocellulose, and the blot was probed with a radiolabeled 1.3-kb EcoRI-PstI fragment from the upstream region of *FKS1*. An autoradiograph of the resulting blot is shown on the left, and the predicted fragment sizes and genomic structures of possible recombinants are shown on the right. (B) The blot depicted in panel A was stripped of the 1.3-kb EcoRI-Pst1 *FKS1* probe and reprobed with a radiolabeled 1.4-kb fragment containing the *hphB* gene, as shown on the diagram at the right side of the figure.
null allele were readily lost or did not produce viable conidia. In fact, in all cases conidia from VFC10 and VFC13 taken from nonselective plates and scored for hygromycin resistance retained resistance (data not shown). Arthroconidia from VFC12, a strain with a FKS1 deletion allele, and VFC13, the control strain, were capable of forming spherules in vitro, and a single round of spherule formation did not decrease the number of hygromycin-resistant CFU (data not shown).

**Heterokaryotic strains with FKS1 null alleles exhibit abnormal swelling of some hyphal elements.** To determine whether the FKS1 gene deletion caused an observable phenotype when present in a heterokaryon, both the null allele-containing heterokaryon strains (VFC12, VFC15, VFC16, and VFC43) and the ectopic insertion control strains (VFC10 and VFC13) were examined microscopically. Arthroconidia were germinated on thin layers of 2× GYE agar medium and allowed to grow at 28°C for 72 h. Hyphae were stained with Calcofluor White and DAPI, mounted on slides, and examined using fluorescent microscopy. Most of the hyphal elements in strains VFC12, VFC15, VFC16, and VFC43 were indistinguishable from wild-type hyphae with respect to hyphal diameter, length, and septation. Upon closer inspection, however, certain regions of the mycelium displayed aberrant conformations, such as blowing out of cell wall elements at hyphal tips and internal compartments (Fig. 5A and B). These aberrant structures were completely absent from mycelia of VFC10 and VFC13, the control strains (Fig. 5C), and of wild-type strains (data not shown). The growth of FKS1 deletion strains and control strains in the presence of caspofungin, the 1,3-β-glucan synthase inhibitor, was also examined, and typical results are shown in Fig. 5D, E, and F. As has been observed previously with related cilofungin treatments in *C. posadasii* studies, caspofungin inhibited growth (compare Fig. 5D, E, and F with 5C) and produced a swelling of hyphal in wild-type strain Silveira as well as VFC12 (ΔFKS1, FKS1WT) and VFC13 (ectopic hphB, FKS1WT) (17).

**DISCUSSION**

Genes encoding the 1,3-β-glucan synthase catalytic subunit have been isolated from several fungi and have been studied best in *S. cerevisiae* experiments (11, 23, 24, 32, 33, 37). β-glucan synthesis has been found to be essential to life in the human pathogenic fungi examined to date (3, 10, 41). Thus, this function is an attractive target for antifungal therapeutics. The 1,3-β-glucan synthase catalytic subunit is targeted by the echinocandin class of antifungal drugs which are effective against several pathogenic fungi in a clinical setting. We sought to determine whether there was a molecular basis for such therapy to be effective in treating patients with coccidioidomycosis. To begin to address this question, we looked for genes in *C. posadasii* that shared similarity to known fungal FKS genes.

In this study, we identified a single gene, FKS1, in *C. posadasii* that showed significant similarity to known fungal FKS genes. Analysis of the predicted protein product, Fks1p, revealed the highest degree of similarity to Fks1p of *P. brasiliensis* (84% identity). *P. brasiliensis* is a dimorphic human pathogenic fungus and, like *C. posadasii*, is a member of the order Onygenales. Fks1p of *A. fumigatus* and FksAp of *A. nidulans* also showed a high degree of similarity to Fks1p of *C. posadasii* (81 and 80%, respectively) and, among fungi whose genomes have been sequenced, are the most closely related to *C. posa-

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**TABLE 1. Growth rates, conidiation, and mitotic stability of ΔFKS1::hphB transformants**

| Strain | Hyg selection in growth medium | Growth rate (mm/day) | Total arthroconidium yield (CFU/mm² × 10⁷) | HygR arthroconidium yield (CFU/mm² × 10⁷) | HygR CFU/total CFU |
|--------|-------------------------------|----------------------|------------------------------------------|------------------------------------------|-------------------|
| VFC12  | –                             | 2.8 ± 0.24           | 50 ± 11                                  | 1.3 ± 0.0                                | 2.7               |
| VFC12  | +                             | 2.7 ± 0.24           | 7.8 ± 1.2                                | 4.0 ± 0.9                                | 52                |
| VFC15  | –                             | 2.8 ± 0.22           | 25 ± 1.4                                 | 0.6 ± 0.1                                | 2.3               |
| VFC15  | +                             | 2.9 ± 0.13           | 12 ± 6.3                                 | 15 ± 2.1                                | 140               |
| VFC16  | +                             | 2.7 ± 0.26           | 20 ± 5.6                                 | 0.6 ± 0.0                                | 3.4               |
| VFC16  | +                             | 2.5 ± 0.10           | 6.6 ± 1.1                                | 6 ± 2.0                                 | 90                |
| VFC43  | +                             | 2.6 ± 0.13           | 28 ± 7.8                                 | 0.6 ± 0.1                                | 2.4               |
| VFC43  | +                             | 2.7 ± 0.13           | 9.4 ± 0.8                                | 11 ± 0.0                                | 120               |
| VFC10  | –                             | 2.8 ± 0.16           | 37 ± 8.5                                 | 32 ± 2.1                                | 87                |
| VFC10  | +                             | 2.8 ± 0.13           | 32 ± 19                                  | 42 ± 4.9                                | 160               |
| VFC13  | –                             | 2.7 ± 0.24           | 34 ± 13                                  | 28 ± 6.4                                | 86                |
| VFC13  | +                             | 2.7 ± 0.19           | 54 ± 18                                  | 58 ± 12                                 | 110               |

The table shows the radial colony growth rates (in millimeters per day) on 2× GYE agar medium at 28°C (Table 1, column 3). Total arthroconidial yields were somewhat higher when VFC12, VFC15, VFC16, and VFC43 were grown without hygromycin selection (50 µg/ml) for transformant strains. Growth rates shown represent the average of three colonies for each strain with standard deviations. Arthroconidia were prepared from two of colonies for each strain (propagated both with and without hygromycin). For each preparation, the total number of viable arthroconidia and hygromycin-resistant arthroconidia, expressed as CFU per square millimeter with standard deviations, was determined and is shown in columns 4 and 5. The ratio of HygR CFU/total CFU is shown in column 6.

The growth of FKS1 deletion strains and control strains in the presence of caspofungin, the 1,3-β-glucan synthase inhibitor, was also examined, and typical results are shown in Fig. 5D, E, and F. As has been observed previously with related cilofungin treatments in *C. posadasii* studies, caspofungin inhibited growth (compare Fig. 5D, E, and F with 5C) and produced a swelling of hyphal in wild-type strain Silveira as well as VFC12 (ΔFKS1, FKS1WT) and VFC13 (ectopic hphB, FKS1WT) (17).
dasii, all belonging to the class Eurotiomycetes. Curiously, the predicted Fksp protein homologs in C. posadasii strain Silveira (this work) and strain C735 (TIGR) contain a unique stretch of 12 glycine residues in their amino termini after amino acid residue 8. These glycine residues are encoded by six GGA codons followed by five GGC codons and a single GGT codon. It will be interesting to see whether this region is present in the sequence of C. immitis strain RS presently being sequenced by The Broad Institute.

Several different approaches have been used to demonstrate the essential nature of FKS genes in other human pathogenic fungi (10, 12, 41). In C. albicans, sequential targeted disruption experiments yielded heterozygotic diploid strains with one FKS1 allele disrupted, but no strains that contained disruptions of both FKS1 alleles were obtained (10). Thompson et al. approached the problem of potential FKS1 essentiality by employing a plasmid that could integrate by homologous recombination in two possible orientations, one that leaves an intact FKS1 gene and one that results in a loss of FKS1 function (41). They found that all transformants obtained had integration events in a single orientation that maintained a functional FKS1 gene; this finding thus supports the idea of its essential nature under the growth conditions examined. Parasexual genetics was applied to determine the essential nature of FKS1 of A. fumigatus (12). A diploid strain was constructed that contained a disrupted FKS1 allele and a wild-type FKS1 allele. Subsequent haploidization of this strain yielded no haploid segregants that contained the disrupted allele.

Initial transformants of C. posadasii obtained by A. tumefaciens-mediated transformation are often heterokaryotic, but homokaryons can be isolated through repeated conidial passage (K. Orsborn, M. J. Orbach, A. Das, M.L Lewis, and J. Galgiani, Abstr. 101st Gen. Meet. Am. Soc. Microbiol., abstr. 48526, 2001). The heterokaryotic nature of primary transformants was considered an advantage in our attempt to disrupt a potentially essential gene. Transformation of C. posadasii to hygromycin resistance using a knockout cassette designed to replace the FKS1 coding sequences with the bacterial hphB gene by a double-crossover event resulted in two classes of transformants. Southern analysis of the isolates from the first class of transformants, after multiple conidial passages, showed patterns of hybridization that indicated the presence of the...
replacement allele at \( FKS1 \) and a wild-type copy of \( FKS1 \) \((\Delta FKS1::hphB, FKS1^{WT})\). When grown in the absence of hygromycin selection, this class gave rise to predominantly Hyg\(^\beta\) arthroconidia. Subsequent DNA hybridization analysis of these segregants showed a loss of the \( FKS1 \) replacement allele, leaving only the wild-type \( FKS1 \) (data not shown). Our interpretation of this result is that a defect in the nuclei containing the null \( FKS1 \) allele results in their rapid loss from a growing mycelial colony in the absence of selection. These results support the hypothesis that these transformants are heterokaryotic for alleles of \( FKS1 \) \((\Delta FKS1::hphB, FKS1^{WT})\) and that the inability to purify strains containing only the replacement allele is due to the requirement of Fks1p for viability. This work also demonstrates the utility of the use of \textit{Agrobacterium}-mediated transformation to generate a gene replacement in \textit{C. posadasii}.

A second class of transformants was obtained that appeared to result from an ectopic insertion of the \( FKS1::hphB \) knockout cassette at random locations in the \textit{C. posadasii} genome. In contrast to the first class of transformants, after initial passaging for purification, these strains exhibited no loss of the Hyg\(^\beta\) marker in the absence of hygromycin selection. This result supports the idea that these strains are homokaryons and that all nuclei present harbored a wild-type \( FKS1 \) allele and an ectopic insertion of the hygromycin resistance marker.

Determining whether \( FKS1 \) is essential during spherule development is more complicated due to the multinucleate character of the developing spherule and the finding that in our hands, strain Silveira only undergoes a single round of spherule development from arthroconidia, with the mature endospore-containing spherules remaining largely intact. In preliminary studies we found no obvious defects in spherules formed by the \( FKS1^{null} \) null allele-containing strains (data not shown). We are thus unable to make a determination about the requirement for Fks1p during spherule development. Measurements of \( FKS1 \) expression using real-time RT-PCR showed that \( FKS1 \) is expressed during the course of this work.

The data presented in this report support the use of 1,3-\( \beta \)-glucan synthase inhibitors in the treatment of coccidioidomycosis. \( FKS1 \) appears to be essential for viability of \textit{Coccidioides} spp., and although we were unable to create a strain homokaryotic for a null allele of \( FKS1 \), the heterokaryotic strain exhibited abnormalities in cell wall integrity and was hypersensitive to inhibitors of 1,3-\( \beta \)-glucan synthase activity. Combination therapy of echinocandins with other classes of antifungals such as polyenes, azoles, or nikkomycin may also be effective approaches to treatment and warrant investigation.

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