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Effects of Depleting the Essential Central Metabolic Enzyme Fructose-1,6-Bisphosphate Aldolase on the Growth and Viability of *Candida albicans*: Implications for Antifungal Drug Target Discovery

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The central metabolic enzyme fructose-1,6-bisphosphate aldolase (Fba1p) catalyzes a reversible reaction required for both glycolysis and gluconeogenesis. Fba1p is a potential antifungal target because it is essential in yeast and because fungal and human aldolases differ significantly. To test the validity of Fba1p as an antifungal target, we have examined the effects of depleting this enzyme in the major fungal pathogen *Candida albicans*. Using a methionine/cysteine-conditional mutant (*MET3-FBA1*/*fba1*), we have shown that Fba1p is required for the growth of *C. albicans*. However, Fba1p must be depleted to below 5% of wild-type levels before growth is blocked. Furthermore, Fba1p depletion exerts static rather than cidal effects upon *C. albicans*. Fba1p is a relatively abundant and stable protein in *C. albicans*, and hence, Fba1p levels decay relatively slowly following *MET3-FBA1* shutoff. Taken together, our observations can account for our observation that the virulence of *MET3-FBA1*/*fba1* cells is only partially attenuated in the mouse model of systemic candidiasis. We conclude that an antifungal drug directed against Fba1p would have to be potent to be effective.

Candida albicans is the major systemic fungal pathogen of humans (5, 35). This fungus is carried as a commensal in the oral and gastrointestinal tracts of many individuals but can cause oral and vaginal infections if normal fungus-host interactions are disturbed. In severely immunocompromised patients, *C. albicans* can cause life-threatening systemic infections (35). A range of virulence attributes, including adhesion, morphogenesis, phenotypic switching, and the secretion of hydrolytic enzymes, contributes to the pathogenicity of this fungus (5, 6, 35).

A limited range of antifungal drugs is available to combat *Candida* infections. Those drugs in routine clinical use include the polyenes, azoles, and echinocandins (36). Polyenes, such as amphotericin B, are thought to bind ergosterol in the fungal plasma membrane, the azoles inhibit ergosterol biosynthesis, and the echinocandins inhibit glucan synthesis (36). The search for novel, broad-spectrum drugs that are fungicidal (rather than fungistatic) and yet exert no significant side effects upon the patient continues. Not surprisingly, this search has focused mainly upon fungus-specific processes, such as cell wall or ergosterol biosynthesis. However, metabolic enzymes have been targeted as potential antibiotic targets in other microbial pathogens (9, 34, 37).

The ability of *C. albicans* to thrive in its mammalian host is due not only to its virulence factors but also to its metabolic flexibility. This fungus can assimilate fermentative or nonfermentative carbon sources, depending upon the host niche it occupies (1, 4, 16, 27). For example, *C. albicans* activates the glyoxylate cycle and gluconeogenesis following phagocytosis by white blood cells, whereas the glycolytic pathway is activated in most fungal cells infecting the kidney (1, 17). These pathways are critical for the virulence of *C. albicans* (1, 27). Therefore, an antifungal drug that selectively inhibits central carbon metabolism might prove an attractive addition to the therapeutic armory.

Fructose-1,6-bisphosphate aldolase (Fba1p) catalyzes the reversible cleavage of fructose-1,6-bisphosphate to dihydroxyacetone phosphate and glyceraldehyde 3-phosphate. We considered Fba1p an attractive antifungal target for several reasons. First, this key enzyme is required for growth on both fermentative and nonfermentative carbon sources. Hence, *FBA1* is an essential gene in *Saccharomyces cerevisiae* (22), and we reasoned that it would also be essential for viability in *C. albicans* and other pathogenic fungi. Second, *FBA1* is a unique gene in *C. albicans*. The *C. albicans* genome sequence contains only one homologue of the *S. cerevisiae FBA1* gene (11, 23; http://genolist.pasteur.fr/CandidaDB). Third, *C. albicans FBA1* exhibits strong sequence similarity to its orthologues in *Schizosaccharomyces pombe*, *Aspergillus nidulans*, and *Neurospora crassa*, suggesting that an antifungal agent directed against *C. albicans* Fba1p might have broad specificity. Fourth, fungal fructose-1,6-bisphosphate aldolases are distinct from human fructose-1,6-bisphosphate aldolases. *C. albicans* Fba1p belongs to the family of class II aldolases found predominantly in fungi and prokaryotes (30). Class II aldolases act as homodimers, catalyzing the aldol cleavage of fructose 1,6 bisphosphate by using a zinc ion as an electrophile (8, 44). In contrast, the human enzyme belongs to the class I aldolases. These operate via a different catalytic mechanism, forming a Schiff base with the dihydroxyacetone moiety of the substrate during catalysis. The sequence of human aldolase is significantly different from

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TABLE 1. *C. albicans* strains

Strain	Parent	Genotype	Source or reference
CAI4	SC5314	*ura3*::λ *imm434*/*ura3*::λ *imm434*	15
RM1000	CAI4	*ura3*::λ *imm434*/*ura3*::λ *imm434*, *his1*::*hisG*/*his1*::*hisG*	50
ARC101	RM1000	*ura3*::λ *imm434*/*ura3*::λ *imm434*, *his1*::*hisG*/*his1*::*hisG*, *FBA1*/*fba1*::*loxP-HIS1-loxP*	This study
ARC102	RM1000	*ura3*::λ *imm434*/*ura3*::λ *imm434*, *his1*::*hisG*/*his1*::*hisG*, *URA3-MET3-FBA1*/*FBA1*	This study
ARC103	RM1000	*ura3*::λ *imm434*/*ura3*::λ *imm434*, *his1*::*hisG*/*his1*::*hisG*, *URA3-MET3-FBA1*/*fba1*::*loxP-HIS1-loxP*	This study
ARC104	RM1000	*ura3*::λ *imm434*/*ura3*::λ *imm434*, *his1*::*hisG*/*his1*::*hisG*, *URA3-MET3-FBA1*/*fba1*::*loxP-HIS1-loxP*	This study
ARC105	RM1000	*ura3*::λ *imm434*/*ura3*::λ *imm434*, *his1*::*hisG*/*his1*::*hisG*, *URA3-MET3-FBA1*/*fba1*::*loxP-HIS1-loxP*	This study
ARC106	RM1000	*ura3*::λ *imm434*/*ura3*::λ *imm434*, *his1*::*hisG*/*his1*::*hisG*, *CIp20-URA3-HIS1*	This study
TY 1	CAI4	*ura3*::λ *imm434*/*ura3*::λ *imm434*, *MET3-URA3*	This study

those of fungal aldolases (30), suggesting that selective inhibitors of fungal aldolases could be identified.

In this study, we have addressed the potential of *C. albicans* Fba1p as an antifungal target. Using well-defined conditional *FBA1* mutants, we have examined the effects of Fba1p depletion upon the growth and virulence of *C. albicans*. We discuss the implications of our findings for the design of antifungal drug screens that target abundant and stable metabolic enzymes.

MATERIALS AND METHODS

Strains and growth media. *C. albicans* strains (Table 1) were grown in YPD (yeast-peptone-dextrose) (45) or synthetic complete (SC) medium lacking methionine and cysteine (24). SC medium was supplemented with 2.5 mM methionine and cysteine for MET3 promoter shutoff assays.

Strain construction. To generate conditional *C. albicans FBA1* mutants, one *FBA1* allele was placed under the control of the *MET3* promoter (7), and the other allele was disrupted using the *lox-HIS1-lox* marker (12). The *URA3-MET3* region from plasmid pURA3-MET3 was PCR amplified with *Pfu* Turbo (Promega; Southampton, United Kingdom) by use of primers MUF-F and MUF-R (Table 2) to generate a *URA3-MET3-FBA1* cassette with 80 bp of homology to the *FBA1* 5′ upstream region and 80 bp of homology to the start of the *FBA1* open reading frame. Similarly, the *loxP-HIS1-loxP* sequence from plasmid pLHL (12) was PCR amplified using primers LHL-F and LHL-R (Table 2) to generate an *fba1*::*HIS1* disruption cassette with 80 bp of flanking homology to the 5′ upstream and 3′ downstream regions of the *FBA1* gene. *C. albicans* strain RM1000 (Table 1) was then transformed with these cassettes as described previously (42) to generate strains ARC101 (*FBA1*/*fba1*) and ARC102 (*MET3-FBA1*/*FBA1*) (Fig. 1). ARC102 was then transformed with the *fba1*::*HIS1* cassette to generate strains ARC103 to ARC105 (*MET3-FBA1*/*fba1*). The genotype of each mutant was confirmed by PCR and Southern blotting (32).

Fructose-1,6-bisphosphate aldolase assays. Fba1p enzyme assays were performed using procedures adapted from reference 41, and the reaction buffer was developed by Maitra and Lobo (29). *C. albicans* cells were washed three times in 1 M KCl and resuspended in 250 μl of a reaction buffer (2 mM EDTA, 2 mM β-mercaptoethanol, 50 mM $KHPO_4$, pH 7.4). Cells were sheared with 150-μl glass beads by use of a Fast Prep FP120 bead beater (Bio 101; Thermo Electron Corp., Cambridge, United Kingdom) and centrifuged at 15,000 × *g* for 5 min at 4°C. Supernatants were stored at −20°C. The rate of Fba1p-catalyzed cleavage of fructose-1,6-bisphosphate to α-glycerophosphate was measured the next morning by coupling of this reaction to the conversion of dihydroxyacetone phosphate to α-glycerophosphate with the concomitant oxidation of NADH to NAD^+, which was monitored at A_{340}. The protein concentrations of cell extracts were determined (47), and mean Fba1p specific activities (nm/min/mg protein) were calculated using two separate extracts assayed in duplicate.

Murine model of systemic candidiasis. The mouse model of systemic candidiasis was used to assess the virulence of *C. albicans* strains (28). Growth of fungal strains in vivo, rather than mouse survival, was assayed. *C. albicans* strains were grown for 18 h at 35°C in SC medium lacking uridine, histidine, methionine, and cysteine. Groups of five immunocompetent female CD1 mice (Charles River, Margate, United Kingdom) were inoculated via the lateral tail vein with 2.5×10^5 cells/mouse, and three control mice received saline alone. This inoculation size was chosen following careful titration experiments to ensure that mice infected with the positive control strain (ARC106) survived until the end of the study (not shown). Mice were monitored over 5 days, after which they were humanely sacrificed. Kidneys were removed postmortem and homogenized in 0.5 ml of water and *C. albicans* tissue burdens determined by viable counting (28). All experimentation was carried out under the terms of the United Kingdom Home Office licenses for research on animals.

TABLE 2. PCR primers

Primer	Use	Sequence (5′–3′)
MUF-F	Amplification of *URA3-MET3-FBA1* cassette	AGTGAGTGAGTGAGTGGTGTGTCATAATAATGAATTGTC TGTTGGTGGTGGTGGTTTGATTAGCACCACCACCATAC ATAACTAATAGGAATTGATTGGATGG
MUF-R	Amplification of *URA3-MET3-FBA1* cassette	TCTTGAGCATAGTCAAACAAGTCTTTGACGTCTTTACCGT AGATAACACCGGATTTACTTAAAACTGCTGGAGGAGC CATGGGGAGGGTATTTACTTTTAAATA
LHL-F	Amplification of *fba1*::*HIS1* cassette	CCCAATCTTCCCGTTCTTTCAAATCAAAAATTTTTCAACC CCCTTTTCTTTATTTCTTTTCTTTTGTTTTTGTTCTACAC CCTCTTCGCTATTACGCCAG
LHL-R	Amplification of *fba1*::*HIS1* cassette	CAACACTGCTATCAAGAATGGTGTTGTCAAGGTCAACTT GGACACTGATTGTCAATATGCTTACTTGACTGGTATCA GAGGCAGATTACCCTGTTATCCCTA
FBA-F3	Diagnosis of *FBA1* and *fba1*::*HIS1* alleles	CAGGCAGAAAGTGGGAGC
FBA-R3	Diagnosis of *FBA1* and *URA3-MET3-FBA1* alleles	CGGCTTTCAACATACCATCA
MET3p-F	Diagnosis of *URA3-MET3-FBA1* allele	ATTGCTGTGGATCACGTGC
HIS-R1	Diagnosis of *fba1*::*HIS1* allele	CACAAGAAGCCTCAACGG
FBA-F4	Amplification of *FBA1* probe for Southern analysis	AGGATCAGTTTTTCCCCGG
FBA-R4	Amplification of *FBA1* probe for Southern analysis	GCTAATCAAACCACCACCACC

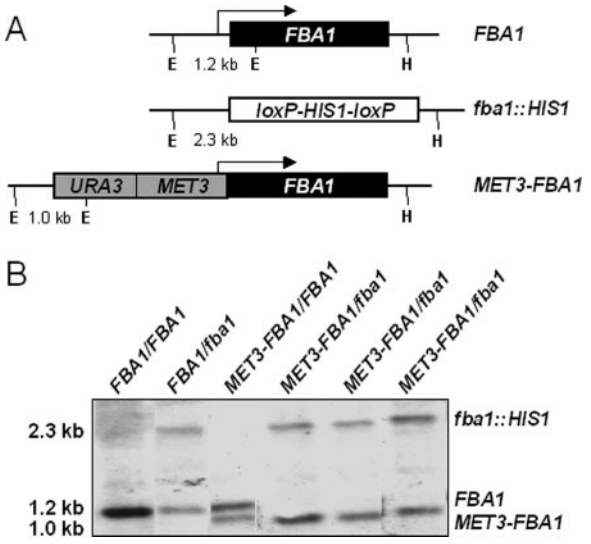

FIG. 1. Construction of methionine-conditional *C. albicans FBA1* mutants. One *FBA1* allele was disrupted by insertional inactivation with the *loxP-HIS1-loxP* cassette, and the other *FBA1* allele was placed under the control of the *MET3* promoter. (A) Structure of the wild-type (*FBA1*), null (*fba1*::*HIS1*), and conditional (*MET3-FBA1*) alleles, with highlighting of the 5′ EcoRI (E)-HindIII (H) fragments detected by Southern blotting. (B) Southern blot of EcoRI-HindIII double-digested genomic DNA from the *C. albicans FBA1* wild type, *FBA1*/*FBA1* (RIM1000); the *FBA1*/*fba1* (ARC101) and *MET3-FBA1*/*FBA1* (ARC102) mutants; and three independent conditional *MET3-FBA1*/*fba1* mutants (ARC103 to ARC105). The restriction fragments corresponding to each allele are indicated.

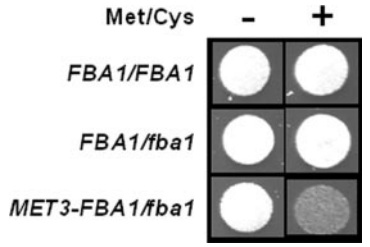

FIG. 2. The conditional *C. albicans FBA1* mutants display a methionine-dependent growth defect on plates. *C. albicans* strains were grown at 30°C for 24 h on SC agar containing (+) or lacking (−) methionine (Met) and cysteine (Cys). Results for *FBA1*/*FBA1* (RIM1000), *FBA1*/*fba1* (ARC101), and *MET3-FBA1*/*fba1* (ARC103) are shown. Strain ARC102 (*MET3-FBA1*/*FBA1*) grew similarly to RIM1000 and ARC101, whereas ARC104 and ARC105 displayed phenotypes similar to that of ARC103 (not shown).

RESULTS

Construction of conditional *C. albicans FBA1* mutants. For fructose-1,6-bisphosphate aldolase (Fba1p) to be considered a promising antifungal target, it should be essential for the growth of fungal pathogens. The *FBA1* gene is essential in *S. cerevisiae* (22) and was predicted to be essential in *C. albicans.* Not surprisingly, therefore, repeated attempts to generate homozygous *fba1*::*HIS1*/*fba1*::*URA3* null mutants in this diploid fungus were unsuccessful. Instead, we constructed conditional *FBA1* mutants to test the essentiality of this gene in *C. albicans*. We used the *C. albicans MET3* promoter, which is tightly repressed by methionine and cysteine (7) and which was used very recently in an effective screen of conserved fungal genes for potential antifungal targets (26). Using a PCR-amplified *URA3-MET3-FBA1* cassette, the first *FBA1* allele was placed under the control of this *MET3* promoter. An *fba1*::*HIS1* cassette was then PCR amplified and used to disrupt the second *FBA1* allele (Fig. 1A). The genotypes of the resulting methionine/cysteine-conditional *MET3-FBA1*/*fba1* mutants were confirmed by PCR diagnosis and Southern blotting (Fig. 1B).

Fba1p is required for the growth of *C. albicans*. To test whether Fba1p is required for the growth of *C. albicans*, first we examined the growth of *MET3-FBA1*/*fba1* cells on solid media. Equivalent numbers of wild-type and mutant cells were spotted onto SC medium plates containing glucose or amino acids as the carbon source and incubated at 30°C. Cells were grown in the presence of methionine and cysteine to repress the expression of the *MET3-FBA1* allele, and control cells were grown in the absence of these amino acids. As expected, all strains exhibited normal growth in the absence of methionine and cysteine (Fig. 2). Also, wild-type cells (*FBA1*/*FBA1*) and the heterozygous mutants (*FBA1*/*fba1* and *MET3-FBA1*/*FBA1*) grew normally in the presence of methionine and cysteine. In contrast, the conditional mutant (*MET3-FBA1*/*fba1*) reproducibly exhibited attenuated growth in the presence of methionine and cysteine (Fig. 2). Similar effects were observed on gluconeogenic carbon sources (not shown). This was consistent with the idea that Fba1p is required for both glycolytic and gluconeogenic growth in *C. albicans*.

Complete growth inhibition was expected for *MET3-FBA1*/*fba1* cells in the presence of methionine and cysteine. However, growth was only partially attenuated on plates. Therefore, to further test the effects of *MET3-FBA1* shutoff, growth in liquid medium was examined. Wild-type, heterozygous, and conditional strains were cultured in SC medium containing or lacking methionine and cysteine (Fig. 3A). The growth of wild-type and heterozygous cells was essentially unaffected by the presence of 2.5 mM methionine and cysteine. However, the growth of the conditional *MET3-FBA1*/*fba1* cells stopped after about 5 hours in the presence of methionine and cysteine. This reinforced the idea that Fba1p is required for the growth of *C. albicans.* The delayed effects of methionine and cysteine upon the growth of *MET3-FBA1*/*fba1* cells, which were observed reproducibly in replicate experiments, probably account for the partial inhibition of growth in plate assays.

Slow depletion of Fba1p levels following *MET3-FBA1* repression. Why was the effect of *MET3-FBA1* repression upon growth not immediate? In *S. cerevisiae*, glycolytic genes are expressed at high levels during fermentative growth, their mRNAs are relatively stable, and glycolytic enzymes are thought to have low turnover rates (18–21, 31). In particular, *FBA1* is expressed at high levels in *S. cerevisiae* (44). The same is probably true in *C. albicans*. Glycolytic mRNAs are expressed at high levels in this pathogen, glycolytic enzymes are abundant, and Fba1p is an abundant protein under all conditions tested (48, 52). Hence, even if *MET3-FBA1* transcription is rapidly repressed following the addition of methionine and cysteine to the growth medium, the *FBA1* mRNA might decay slowly, allowing Fba1p synthesis to continue for some time thereafter, and the Fba1p protein might be relatively stable. We predicted, therefore, that the continued presence of Fba1p

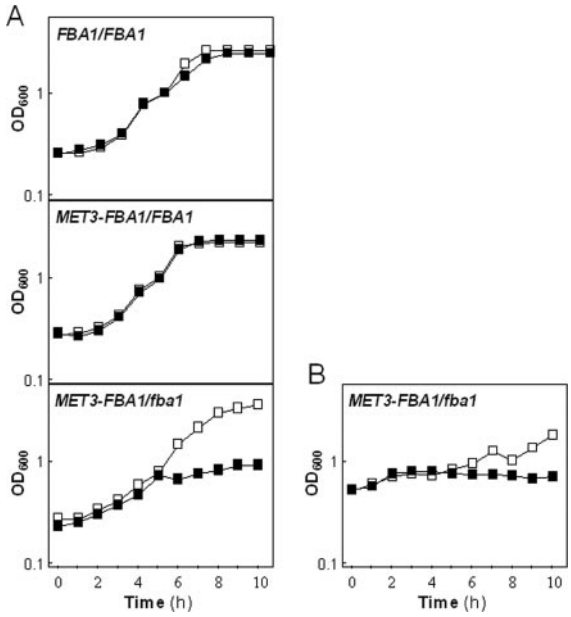

FIG. 3. Behavior of the conditional *C. albicans FBA1* mutants in liquid media. (A) Growth of the conditional *C. albicans FBA1* mutants continues for hours after the addition of methionine and cysteine. *C. albicans* strains were grown at 30°C in SC medium containing (black squares) or lacking (white squares) methionine and cysteine. Results for *FBA1*/*FBA1* (RIM1000), *MET3-FBA1*/*FBA1* (ARC102), and *MET3-FBA1*/*fba1* (ARC103) are shown. In replicate experiments, strain ARC101 (*FBA1*/*fba1*) grew similarly to RIM1000 and ARC102, whereas ARC104 and ARC105 grew similarly to ARC103 (not shown). (B) *MET3-FBA1* shutoff is static, not cidal. *C. albicans* ARC103 cells (*MET3-FBA1*/*fba1*) from the culture containing methionine and cysteine were subcultured into fresh SC medium containing (black squares) or lacking (white squares) methionine and cysteine, and their growth was monitored. Similar data were obtained in replicate experiments. OD_{600}, optical density at 600 nm.

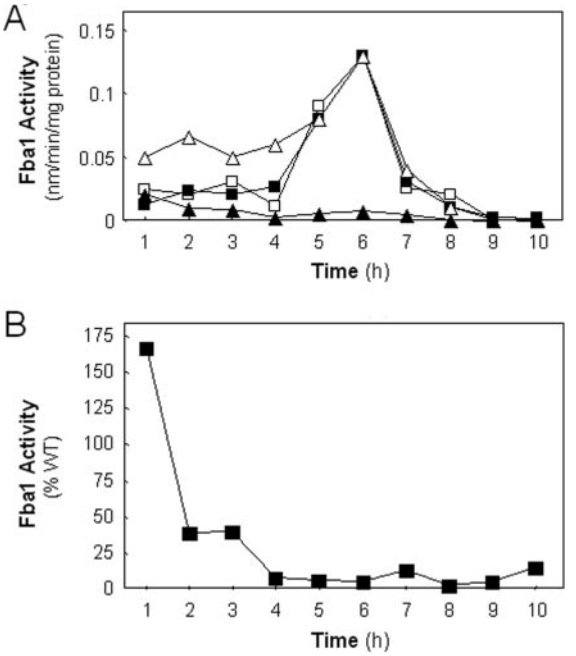

FIG. 4. Depletion of fructose-1,6-bisphosphate aldolase levels in the conditional *C. albicans FBA1* mutants. (A) Fba1p assays were performed on extracts prepared from *C. albicans* RM1000 and ARC103 cells grown at 30°C in SC medium containing or lacking methionine and cysteine. Results for SC medium with *FBA1*/*FBA1* (white squares), SC medium containing methionine and cysteine with *FBA1*/*FBA1* (black squares), SC medium with *MET3-FBA1*/*fba1* (white triangles), and SC medium containing methionine and cysteine with *MET3-FBA1*/*fba1* (black triangles) are shown. Strains were pregrown overnight in SC medium lacking methionine and cysteine and inoculated at similar cell densities at the beginning of the experiment (optical density at 600 nm, ~0.25). (B) Reduction in the relative levels of active Fba1p in the conditional *C. albicans MET3-FBA1*/*fba1* cells (ARC103) compared with levels in wild-type (WT) cells (RM1000) following the addition of methionine and cysteine to both cultures. Similar data were obtained in two independent experiments.

in *MET3-FBA1*/*fba1* cells for a significant period after methionine and cysteine addition might account for the delayed effects upon the growth of *C. albicans*.

To test this, we measured the levels of active Fba1p enzyme during *MET3-FBA1* shutoff experiments. Wild-type and conditional *MET3-FBA1*/*fba1* cells were inoculated into SC medium containing or lacking methionine and cysteine, and Fba1p enzyme assays were performed at various times thereafter (Fig. 4A). The presence of methionine and cysteine had little effect upon Fba1p levels in wild-type cells. Fba1p levels increased during the exponential growth phase, reaching a peak after 6 hours (Fig. 4A), just before cells entered diauxie (Fig. 3A), after which they declined rapidly. This is consistent with the expression patterns of other glycolytic genes in *C. albicans* (2, 39). A similar pattern was observed in *MET3-FBA1*/*fba1* cells in the absence of methionine and cysteine, except that Fba1p levels were initially higher in early exponential phase (Fig. 4A). In contrast, when *MET3-FBA1*/*fba1* cells were exposed to methionine and cysteine, Fba1p levels decreased with time, approaching zero only after 4 to 5 hours. This trend is more obvious when Fba1p levels in *MET3-FBA1*/*fba1* cells are expressed relative to those in wild-type cells under the corresponding conditions (i.e., in the presence of methionine and cysteine) (Fig. 4B). Therefore, Fba1p remains present for some hours after *MET3-FBA1* repression.

Relationship between Fba1p levels and growth. These experiments address the relationship between Fba1p levels and growth in *C. albicans*. To explore this further, we compared relative Fba1p levels with the doubling time of *C. albicans* during exponential growth phase, combining data from two independent experiments (Fig. 5). It is evident that Fba1p levels must drop to below 15% of wild-type levels before the growth of *C. albicans* is inhibited significantly. For near-complete growth inhibition (<5% of wild-type levels), Fba1p levels must fall to below 4% of those for wild-type cells.

Fba1p depletion stops growth but does not kill *C. albicans*. We tested whether Fba1p depletion merely inhibits growth or whether it kills *C. albicans* cells. To achieve this, *fba1*/*MET3-FBA1* cells were grown for 10 hours in medium containing methionine and cysteine. These cells, which had stopped growing (Fig. 3A), were harvested, washed, and resuspended in fresh SC medium. Equivalent numbers of cells were used to inoculate SC medium containing methionine and cysteine and SC medium lacking these amino acids. Even after prolonged repression, *fba1*/*MET3-FBA1* resumed normal growth in me-

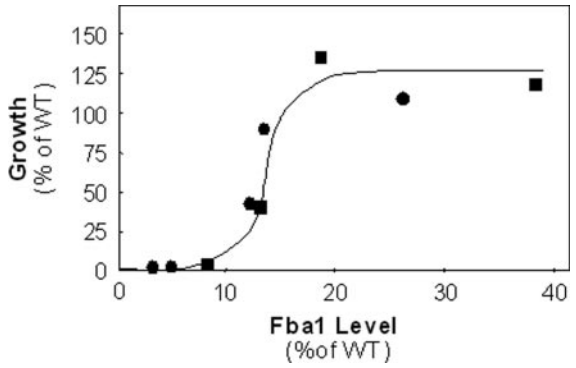

FIG. 5. Relationship between level of active Fba1p and growth of *C. albicans*. *C. albicans* RM1000 (wild type [WT]) and ARC103 (*MET3-FBA1*/*fba1*) were grown in SC medium containing methionine and cysteine at 30°C, and their growth was monitored (optical density at 600 nm). The levels of active Fba1p were measured at various times during exponential growth, and Fba1p levels in the mutant were expressed as percentages of those in wild-type cells. The doubling times of the cultures were monitored at the time of Fba1p measurement, and those of mutant cells were expressed as percentages of those of wild-type cells. Results for experiment 1 (black squares) and experiment 2 (black circles) are shown. These doubling times reflect the rate of generation of new biomass and hence the biochemical flux to "growth."

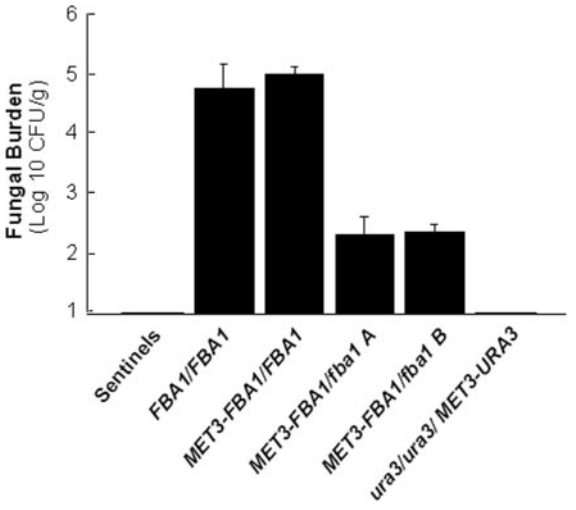

FIG. 6. The virulence of the conditional *C. albicans FBA1* mutants is attenuated in the mouse model of systemic candidiasis. Fungal burdens were measured in the kidneys of mice humanely sacrificed 5 days after injection with 2.5×10^5 *C. albicans* cells. Results for *FBA1*/*FBA1* (ARC106), *MET3-FBA1*/*FBA1* (ARC102), *MET3-FBA1*/*fba1 A* (ARC103), *MET3-FBA1*/*fba1 B* (ARC104), and the *ura3*/*ura3*/*MET3-URA3* control (TY 1) are shown. Sentinels, no fungal cells injected.

dium lacking methionine and cysteine (Fig. 3B). The viability of *fba1*/*MET3-FBA1* cells following prolonged incubation in medium containing methionine and cysteine was confirmed by plate assays (not shown). This suggests that Fba1p depletion has a static rather than a cidal effect upon *C. albicans*, although it should be noted that *C. albicans* cells can survive some "cidal" antifungals even after prolonged treatment.

We tested whether Fba1 depletion might act synergistically with an azole antifungal to inhibit the growth of *C. albicans*. However, we did not observe a reduction in MIC when *fba1*/*MET3-FBA1* cells were exposed to fluconazole in the presence of methionine and cysteine, compared with results obtained with control cultures lacking these amino acids or containing *FBA1*/*MET3-FBA1* cells (not shown).

Virulence of conditional *FBA1* mutants. To further examine the potential of Fba1p as an antifungal target, we tested the virulence of *C. albicans fba1*/*MET3-FBA1* cells. We used the mouse model of systemic candidiasis, which has been used frequently to determine potential virulence factors and also to analyze host-pathogen interactions (13, 28, 33). Assays were designed to assess the growth of the fungus in vivo rather than the speed with which it kills the host. Mice were injected with inocula containing either saline alone (sentinels), wild-type cells (*FBA1*/*FBA1*), heterozygous cells (*MET3-FBA1*/*FBA1*), two different conditional mutants (*MET3-FBA1*/*fba1*), or a *ura3*/*ura3*/*MET3-URA3* control strain (TY 1) (Table 1). Fungal burdens were determined for several tissues after 5 days (Fig. 6). The genotypes of the *C. albicans* strains isolated from animals were reconfirmed by diagnostic PCR (data not shown). As expected, heavy fungal burdens were observed for the positive controls (wild-type and heterozygous cells), and no detectable fungi were observed in the kidney for the negative controls (sentinels and *ura3*/*ura3*/*MET3-URA3* cells). This confirmed that *MET3-URA3* expression levels in vivo are insufficient to support the growth and dissemination of *C. albicans* following a bloodstream infection. The fungal burdens generated by both of the conditional *MET3-FBA1*/*fba1* mutants were at least 2 orders of magnitude lower than those generated by the positive controls (Fig. 6), indicating repression of the *MET3-FBA1* allele in vivo. Nevertheless, these strains did establish infections in mice. The *URA3* gene was present at the *FBA1* locus in both *MET3-FBA1*/*FBA1* and *MET3-FBA1*/*fba1* cells. Therefore, the reduced fungal burden of the conditional mutant was not the result of *URA3* position effects (3, 46). These data indicate that the virulence of *MET3-FBA1*/*fba1* cells is partially, but not completely, attenuated.

DISCUSSION

The aim of this project was to test the potential of *C. albicans* Fba1p as an antifungal target. Several observations support the notion that Fba1p is an attractive antifungal target.

First, Fba1p is required for the growth of *C. albicans* as well as in *S. cerevisiae* (22). The observation that *MET3-FBA1*/*fba1* cells ceased growing after prolonged exposure to methionine and cysteine is entirely consistent with the idea that *FBA1* is an essential gene in *C. albicans* (Fig. 2 and 3). Second, fungal fructose-1,6-bisphosphate aldolases exploit a catalytic mechanism different from that of the human enzymes (8, 30, 44). Third, there is significant sequence conservation among fungal aldolases, and yet the primary structures of the fungal and human enzymes differ significantly. These observations suggest that it might be possible to develop broad-spectrum antifungal agents that selectively inhibit fungal aldolases.

On the other hand, other observations reduce the attraction of Fba1p as an antifungal target. First, Fba1p depletion appears to exert static rather than cidal effects upon *C. albicans* (Fig. 3B). Therefore, an antifungal drug directed against Fba1p might be expected to prevent the growth of *C. albicans* rather than kill fungal cells infecting a patient. An

anti-Fba1p drug might provide effective protection against fungal infection but would be unlikely to cure patients with systemic candidiasis.

Second, Fba1p levels must be reduced by at least 95% to prevent the growth of *C. albicans* (Fig. 5). This reflects the extent to which Fba1 contributes to the regulation of the metabolic flux (flow of substrates and metabolic intermediates) to the generation of new biomass. The flux control coefficient defines the extent to which a specific enzyme regulates the flux through a specific metabolic pathway (14). Clearly, in *C. albicans*, fructose-1,6-bisphosphate aldolase has a low control coefficient with respect to the metabolic flux from glucose to biomass under the growth conditions examined (Fig. 5). This is similar to the situation for glycolytic enzymes in *S. cerevisiae*. In budding yeast, glycolytic enzymes have low control coefficients, indicating that their contribution to the regulation of glycolytic flux during fermentative growth is minimal (10, 38, 43). Instead, most control is exerted at the level of glucose import (51). This suggests that an anti-Fba1p drug would have to be potent to block the growth of *C. albicans*.

Fba1p is an abundant and stable enzyme in *C. albicans*. Our proteomic analyses of *C. albicans* have revealed that Fba1p is an abundant protein, constituting about 2% of the total soluble proteins with pIs between 4 and 7 (52). Also, following the addition of methionine and cysteine to *MET3-FBA1*/*fba1* cells, it takes at least 4 hours for Fba1p levels to be reduced to below 10% of wild-type levels (Fig. 4). This represents an effective half-life of about 45 min, which is close to the doubling time of the cells (about 50 min). We used the *MET3* promoter because it is rapidly and effectively repressed by methionine and cysteine (7, 25, 26). However, once *MET3-FBA1* transcription has been repressed, Fba1p depletion is dependent upon the turnover of *FBA1* mRNA and Fba1p protein as well as the dilution rate during cell doubling. The slow depletion of *C. albicans* Fba1p following *MET3-FBA1* shutoff (Fig. 4B) is consistent with the behavior of glycolytic mRNAs and enzymes in *S. cerevisiae*, which are generally stable (18–21, 31). The relatively low decay rate for Fba1p, combined with the need for almost complete loss of Fba1p before *C. albicans* growth is effectively inhibited, probably accounts for our observation that the growth of *MET3-FBA1*/*fba1* cells is only partially attenuated in the mouse model of systemic candidiasis (Fig. 6).

Taken together, our observations might suggest that the Fba1p is not an attractive antifungal target. However, from a kinetic perspective, genetic depletion of Fba1p is likely to differ significantly from pharmacological inhibition of Fba1p. An antifungal drug inhibits its target within a significantly shorter time scale than the depletion of the target following transcriptional repression. A classic approach to the identification of novel antifungal targets involves the screening of libraries of conditional mutants in which specific genes have been placed under the control of a regulatable promoter (26, 40). This type of screen has great potential for the identification of novel essential functions that are expressed at low abundance and/or rapidly turned over in *C. albicans*. However, like most screens, it is likely to miss some valid targets. In particular, this approach is likely to miss those essential genes that encode abundant, stable proteins. One solution might be to adapt the screen by artificially accelerating the degradation of target proteins in conditional mutants by including an amino-terminal-protein-destabilizing element along with the regulatable promoter (49).

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