Requirement for the *Candida albicans* *FAS2* gene for infection in a rat model of oropharyngeal candidiasis

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The virulence of *Candida albicans* strains deficient in fatty acid synthase activity by virtue of disruption/deletion of the *FAS2* gene was examined in a rat model of oropharyngeal candidiasis. The *FAS2* alleles of *C. albicans* CA14 (Δ ura3::imm434/ Δ ura3::imm434) were sequentially disrupted with a cassette that included a portion of *FAS2* from which a 984 bp fragment containing the FAS condensing reaction domain was deleted and replaced with *hisG-URA3-hisG* sequences. Verification of fatty acid synthase inactivation was obtained from assays of enzyme activity. Strains in which a single allele was disrupted (CFD1 and CFD3) exhibited an approximately 20% reduction in activity, when compared to wild-type. In addition, fatty acid synthase activity was abolished in a *FAS2* null mutant strain (CFD2), and growth of CFD2 occurred only when the growth medium was supplemented with Tween 40 and certain fatty acids. Strain CFD2 was avirulent in the rat model, indicating that fatty acid synthase activity is required for *C. albicans* oropharyngeal infection. Strains with a single *FAS2* allele disruption colonized the oral cavity, but the number of cells recovered from infected animals was approximately fivefold less than for the parental strain. The results suggest that FAS may be exploited as a possible target for the development of new antifungal agents.

Keywords: *Candida albicans*, *FAS2*, null mutants, avirulence, oropharyngeal

INTRODUCTION

Candida albicans is an opportunistic fungal pathogen of increasing medical significance, particularly in immunocompromised individuals. The organism promotes a wide array of infections ranging from minor irritations to systemic disease (Odds, 1988). Furthermore, *C. albicans* is responsible for the largest percentage of fungal oropharyngeal infections in the AIDS patient (Holmberg & Meyer, 1986). A number of potential virulence factors have been identified, including germ-tube formation (Cutler, 1991; De Bernardis *et al.*, 1993), protease production (Ruchel *et al.*, 1992) and phenotypic switching (Soll, 1992). Detailed investigations, however, concerning these processes, as well as analysis of other genes that may specify products required in pathogenesis, have been restricted until recently by the lack of suitable methods to perform certain molecular manipulations. In this regard, development and use of a targeted gene disruption scheme for *C. albicans* (Fonzi & Irwin, 1993) has led to the definition of genes required for maximal infectivity

(Ghannoum *et al.*, 1995; Becker *et al.*, 1995), and perhaps more importantly, may lead to identification of promising targets to exploit in the search for new antifungal drugs (Bulawa *et al.*, 1995).

One such potential target is fatty acid synthase. This enzyme directs the synthesis of fatty acids required for the assembly of cellular membrane-containing structures, and is required for cell growth in the absence of fatty acids supplied from an exogenous source. The fungal enzyme comprises two polypeptides, α and β , specified by the *FAS2* and *FAS1* genes, respectively (Kuziora *et al.*, 1983; Schweizer *et al.*, 1987). The enzyme is a hexamer and is multifunctional, directing the seven component activities required in fatty acid synthesis (Stoops *et al.*, 1978; Schweizer *et al.*, 1987). In contrast, mammalian fatty acid synthase is a homodimer, with the polypeptide, termed α , configured head to tail (Witkowski, *et al.*, 1991; Smith, 1994). While homologies exist between fungal and mammalian fatty acid synthases, particularly within catalytic domains (Siggaard-Andersen, 1993), their spacing

and linear order, as well as the overall structure of the respective enzymes, are clearly distinct (Schweizer *et al.*, 1987; Smith, 1994). Thus, it is possible that compounds differentially affecting the respective fatty acid synthase might be found, and a recent report has described such agents (Broedel *et al.*, 1996). It remains to be demonstrated, however, that the enzyme is necessary for infection. To address this issue appropriate *C. albicans* *FAS2* mutant strains have been constructed and used in experiments examining the virulence of mutants in a rat oral model of candidiasis.

METHODS

Strains and culture conditions. *C. albicans* strains used in this study are listed in Table 1. The strains were maintained on YEPD medium (1%, w/v, yeast extract; 2%, w/v, peptone; 2%, w/v, glucose; and 1.5%, w/v, agar). Defined medium consisted of 2% (w/v) glucose supplemented with 0.7% (w/v) amino-acid-free yeast nitrogen base. Media were supplemented with uridine (25 µg ml⁻¹) as required. Ura⁻ auxotrophs were selected on medium containing 5-fluoroorotic acid as described by Boeke *et al.* (1984). Strain CFD2 (*FAS2* null mutant) was grown on YEPD supplemented with 1% (v/v) Tween 40, 0.01% (w/v) myristic acid and 0.01% (w/v) stearic acid. For growth-curve determination, overnight cultures were grown in YEPD and in some instances were supplemented with fatty acids as above. Subsequently, aliquots were removed, and diluted in fresh medium to an OD₆₀₀ of 0.1. Cultures were then incubated at 30 °C on a rotary shaker and, at appropriate time intervals, samples were removed and the OD₆₀₀ was determined.

DNA manipulations. All restriction endonuclease digestions were performed under conditions recommended by the manufacturer. Plasmid purification, agarose gel electrophoresis, DNA fragment isolation and Southern blots were performed as described by Sambrook *et al.* (1989). *C. albicans* DNA was isolated as reported by Sherman *et al.* (1986). Probe DNA was labelled with [α -³²P]dCTP by use of a random prime labelling kit (Stratagene).

Plasmid and strain construction. Plasmid pG10a-1, which contains most of *C. albicans* *FAS2* on a 7.9 kb *SalI* fragment (Southard & Cihlar, 1995), was digested with *PstI* and *BamHI* to release a 2.3 kb internal fragment of *FAS2* containing the β -ketoacyl synthase domain (Southard & Cihlar, 1995). The fragment was recovered following agarose gel electrophoresis and then subcloned into compatible sites of the vector, pUC118.

The resulting plasmid, designated pCFU-1, was digested with *EcoRV* and *BglII*, deleting the condensing reaction domain within a 984 bp fragment. The remaining plasmid fragment was recovered and ligated to a 4.0 kb *BamHI*-*PvuII* *bisG*-*URA3-bisG* cassette obtained from plasmid p5921 (Fonzi & Irwin, 1993) to create plasmid pCFU-2. After recovery, pCFU-2 was digested with *PstI* and *BamHI* releasing a DNA fragment containing the *FAS2* gene deletion.

Sequential transformation of strain CAI4 with the constructed cassette was performed as described by Fonzi & Irwin (1993). First, strain CFD1 was obtained as a single *FAS2* allelic disruption by selection of Ura⁺ transformants. Subsequently, a Ura⁻ derivative, CFD1' (*FAS2*/ Δ *fas2*) was isolated by selection on medium containing 5-fluoroorotic acid (Boeke *et al.*, 1984). Finally, CFD2 was isolated as a *FAS2* null mutant after a second round of transformation with the same cassette. A single copy of the wild-type allele was restored by transformation of strain CFD2 with *PstI*- and *BglII*-digested pCFU-1. The desired strain, termed CFD3, was then selected on YNB agar plates where growth of either CFD2 or URA⁻ colonies was precluded by omission of fatty acid and uridine supplements. The strategy for CFD1 and CFD3 construction ensured that the disruption of *FAS2* was not in the same allele in both strains.

Measurement of fatty acid synthase activity. Fatty acid synthase activity was assayed as described elsewhere (Lynen, 1969; McElhane-Feser & Cihlar, 1994). Reaction mixtures (1 ml) contained (final concentrations): 0.4 M potassium phosphate, pH 7.3, containing 0.125 µM dithiothreitol, 50 µM acetyl-CoA, 100 µM NADPH, and crude enzyme preparation. Following incubation at 37 °C for 5 min, malonyl-CoA containing 0.5 µCi [2-¹⁴C]malonyl-CoA [Amersham; 58.8 mCi mmol⁻¹ (2.176 GBq mmol⁻¹)] was added to a final concentration of 100 µM. Reactions were terminated after 10 min by the addition of 30 µl 60% (v/v) perchloric acid and 1 ml absolute ethanol. Fatty acids were extracted four times with light petroleum (b.p. 30–75 °C), extracts were combined and dried in scintillation vials, and finally solubilized in 3a70B scintillation fluid (Research Products International). Radioactivity was measured in a Beckman LSC-230 liquid scintillation counter and enzyme activity was expressed in units (mg protein)⁻¹ (1 unit is the amount of enzyme required to catalyse incorporation of 1 nmol [¹⁴C]malonate into fatty acid min⁻¹ under the conditions of the assay).

Rat model of oropharyngeal candidiasis. The rat model of oropharyngeal candidiasis has been described previously (Meitner *et al.*, 1990; Cole *et al.*, 1995). In the present investigation, weanling, specific pathogen-free Sprague-Dawley

Table 1. *C. albicans* strains used in this study

Strain	Parent	Genotype	Reference
SC5314		Clinical isolate	Gillum <i>et al.</i> (1984)
CAI4	SC5314	Δ <i>ura3::imm434</i> / Δ <i>ura3::imm434</i>	Fonzi & Irwin (1993)
CFD1	CAI4	Δ <i>fas2::bisG-URA3-bisG</i> / <i>FAS2</i> ; Δ <i>ura3::imm434</i> / Δ <i>ura3::imm434</i>	This work
CFD1'	CFD1	Δ <i>fas2::bisG</i> / <i>FAS2</i> ; Δ <i>ura3::imm434</i> / Δ <i>ura3::imm434</i>	This work
CFD2	CFD1'	Δ <i>fas2::bisG</i> / Δ <i>fas2::bisG-URA3-bisG</i> ; Δ <i>ura3::imm434</i> / Δ <i>ura3::imm434</i>	This work
CFD3	CFD2	<i>FAS2</i> / Δ <i>fas2::bisG-URA3-bisG</i> ; Δ <i>ura3::imm434</i> / Δ <i>ura3::imm434</i>	This work

rats, 19–21 d old, identified by litter, were obtained commercially (Charles River Laboratories). To ensure that animals were free of endogenous *C. albicans*, oral swabs were taken and plated onto YEPD and/or Sabouraud agar. Subsequently, hyposalivation was achieved by ligation of the parotid ducts and removal of the submandibular and sublingual salivary glands.

Oral infection was initiated using a cotton-tipped applicator saturated with 10^6 c.f.u. of an actively-growing culture of the appropriate *C. albicans* strain. During the course of the experiment rats were fed diet NIH 2000 containing 56% (w/v) sucrose, and sucrose (10%, w/v) was added to the drinking water. Animals were killed 7 d after inoculation. The mandible was then hemisected and removed to 5 ml sterile saline. Adherent organisms were dislodged by sonication, and the level of colonization with *C. albicans* was determined by plating samples obtained from each animal onto Sabouraud agar supplemented with streptomycin ($300 \mu\text{g ml}^{-1}$) or Sabouraud agar supplemented with 1% (v/v) Tween 40, 0.01% (w/v) stearic acid, 0.01% (w/v) myristic acid, and streptomycin ($300 \mu\text{g ml}^{-1}$). Colonies were quantified after incubation of plates overnight at 30°C .

RESULTS

C. albicans *FAS2* null mutant construction by sequential disruption

A strategy to construct a homozygous disruptant of *C. albicans* *FAS2* was designed utilizing the 'URA-blaster'

Fig. 1. Southern blot analysis of *C. albicans* *FAS2* disrupted/deleted strains. Genomic DNA from individual strains was digested with *Bgl*II, fractionated on a 1% agarose gel and blotted to a nylon membrane. The filter was hybridized with a 7.9 kb *Sal*I fragment containing the *C. albicans* *FAS2* strain. Genomic DNA from: lane 1, CAI4 (wild-type); lane 2, CFD1 (single *FAS2* allele disruption); lane 3, CFD1' (single *FAS2* allele deletion); lane 4, CFD2 (*FAS2* null mutant, one allele disruption, one allele deletion); lane 5, CFD3 (single *FAS2* allele disruption, restoration of second allele).

Fig. 2. Growth analysis of *C. albicans* strains CAI4, CFD1, CFD2 and CFD3. Strains were grown in YEPD supplemented with fatty acids. Overnight cultures of each strain were diluted into fresh medium and cultures were then incubated at 37°C . Samples were removed at the indicated timepoints. $\circ$, CAI4; $\bullet$, CFD1; $\square$, CFD2; $\blacksquare$, CFD3. The growth characteristics of CAI4, CFD1 and CFD3 when strains were grown without medium supplementation were identical to those depicted.

cassette reported by Fonzi & Irwin (1993). The cassette was engineered to introduce a 984 bp deletion in the condensing reaction domain of *FAS2* as described in Methods. *C. albicans* CAI4 (Fonzi & Irwin, 1993), a derivative of the clinical isolate *C. albicans* SC5314 (Gillum *et al.*, 1984), served as the parental strain. The *FAS2* null mutant, designated CFD2, was generated by sequential disruption of each allele. Strain CFD3 contained a reconstructed *FAS2* allele and was included in subsequent analyses to ensure that phenotypic traits attributed to CFD2 were the result of mutation of *FAS2* and not due to other alterations that may have been introduced during CFD2 construction. In addition, since different *FAS2* alleles were disrupted in strains CFD1 and CFD3, any allelic effects of single disruption could be detected. Southern blot analysis confirmed the expected recombination patterns (Fig. 1). Briefly, genomic DNA of the CAI4 parent has two *Bgl*II fragments of 3.9 and 3.7 kb containing *FAS2* sequences (Fig. 1, lane 1). CFD1 (Fig. 1, lane 2) showed disruption of a single *FAS2* allele in which two new *Bgl*II fragments (7.7 and 3.1 kb) were present along with the wild-type pattern of the undisrupted allele. Both *FAS2* alleles of CFD2 have been altered (Fig. 1, lane 4), as shown by the disappearance of all wild-type allele *Bgl*II fragments. Finally, one *FAS2* allele has been reconstructed in CFD3 (Fig. 1, lane 5), demonstrated by the reestablishment of the original 3.9 and 3.7 kb *Bgl*II fragments.

After construction, the growth characteristics of the strains were examined. CFD2 does not grow either in liquid or on solid medium without fatty acid supplementation, while strains CFD1 and CFD3 showed no such requirements. The growth rate of CFD1 and CFD3 in unsupplemented medium was essentially identical to that of the parental strain (Fig. 2). The growth rate of CFD2,

Fig. 3. Fatty acid synthase activity in *C. albicans* *FAS2* mutants. The indicated strains were grown in YEPD medium lacking (-FA) or supplemented (+FA) with fatty acids. Data are expressed as a percentage of the fatty acid synthase activity in the parental strain (CAI4) grown in the absence of fatty acids.

Fig. 4. Comparison of infection with *C. albicans* strains SC5314, CFD1, CFD2 and CFD3 in desalivated rats. Circles represent c.f.u. recovered from individual animals. The mean is designated by a horizontal bar. * *C. albicans* was observed in only two of the five animals infected with CFD2.

when provided with fatty acids, showed no significant differences from the wild-type (Fig. 2). The yeast-to-hyphal transition of all mutant strains occurred normally when induced by temperature shift (data not shown).

Fatty acid synthase activity of mutant strains

In order to examine the effect of *FAS2* disruption on fatty acid synthase, the activity of the enzyme from all strains was assayed (Fig. 3). Strain CFD2 had no detectable fatty acid synthase activity when grown in medium supplemented with fatty acids, and the strain does not grow without such additions. Both strains disrupted in only a single *FAS2* allele (CFD1 and CFD3) maintained approxi-

mately 80% of wild-type activity when grown on a medium without fatty acid supplementation. There was no significant difference in fatty acid synthase activity in cells grown in the presence of fatty acids in the case of CAI4, CFD1 or CFD3.

Avirulence of CFD2 in the rat model of oropharyngeal candidiasis

FAS2 mutant strains were tested for the ability to establish infection in the rat model of oropharyngeal candidiasis. The results depicted in Fig. 4 demonstrate that the ability of CFD2 to colonize the oral cavity is severely impaired. Extremely low numbers of CFD2 were recovered from only two of five inoculated animals. In contrast, CFD1 and CFD3 established infection and were readily recovered from animals at the termination of the experiment. The extent of colonization for these strains, however, was approximately fivefold lower than that observed for the parental strain SC5314. CAI4 was not used as a control in these experiments as it has been demonstrated previously that deletion of *URA3* renders the strain avirulent in the rat model (Cole *et al.*, 1995).

DISCUSSION

The results demonstrate that a *C. albicans* *FAS2* null mutant strain, CFD2, has been obtained. CFD2 was unable to grow without an exogenous supply of fatty acids, and fatty acid synthase was undetectable in the strain. However, the growth rate of CFD2, when supplied with fatty acids, was comparable to that of the parental strain. Finally, Southern blot analysis confirmed mutation of both *FAS2* alleles. In contrast, strains with disruption of either of the *FAS2* alleles (CFD1 and CFD3), when grown without an exogenous fatty acid source, retained approximately 80% of wild-type fatty acid synthase activity. The growth rate of these strains was not adversely affected by this level of loss of enzyme activity. Addition of fatty acids to the growth medium resulted in only a slight reduction of enzyme activity in the case of strains disrupted in one allele of the *FAS2* gene, in comparison to the approximately 20% drop in wild-type activity under the same conditions.

Strain CFD2 was not effective in establishing infection in the rat model of oropharyngeal candidiasis, indicating that fatty acid synthase activity is required for colonization and growth. No systematic studies concerning the level of fatty acids in the rat oral cavity have been reported, and thus it is not possible to speculate concerning the level of free fatty acids that might be available to mutant strains. Similarly, conclusions concerning the ability of CFD2 to colonize at other locations cannot be drawn from the results, and such information will come only from direct experiments. It is clear, however, that the presence of free fatty acids in the oral cavity or their supply through the activity of secreted phospholipases does not compensate for the lack of fatty acid synthase activity in CFD2. On the other hand, strains CFD1 and CFD3 were capable of promoting infection despite a 20% reduction in fatty acid

synthase activity. Paradoxically, the number of c.f.u. recovered from rats infected with CFD1 and CFD3 was approximately fivefold less than noted for the wild-type despite the fact that no significant differences in the *in vitro* growth rate of mutant and parental strains were observed. It is, of course, possible that CFD1 and CFD3 have a slower *in vivo* growth rate, which would account for reduced recovery. Alternatively, it is possible that initial colonization does not occur as efficiently with these two strains.

Most importantly, the results suggest that fatty acid synthase may be a valid target for the development of antifungal agents. While the enzyme is ubiquitous, significant structural differences exist between the mammalian and fungal enzyme, suggesting that compounds with differential activity might be found. In this regard, the effect of modifications to cerulenin, an antilipogenic agent that specifically and irreversibly inhibits fatty acid synthase by inhibition of the condensing reaction (Omura, 1981), on activity of the human and *C. albicans* enzyme was investigated (Broedel *et al.*, 1996). Alterations including halogen, methoxy or trifluoromethyl group decoration of the cerulenin molecule resulted in inhibitory activity against fungal fatty acid synthase, but no detectable activity against the human counterpart. Similar results were noted with a compound containing an ether linkage between the terminal phenyl group and the keto-epoxide moiety of phenylcerulenin (Broedel *et al.*, 1996). In addition, preliminary data presented in the same study detected three as yet unidentified natural products showing differential inhibitory activity. The validation of fatty acid synthase as a potential target in the oropharyngeal model should lead to studies to test the *in vivo* efficacy of agents that are active against fatty acid synthase. The results do not provide evidence that fatty acid synthase will be a suitable target in other models of candidiasis or for infections mediated by other fungi. Experiments are in progress that will address whether fatty acid synthase will be a target of general significance.

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