Virulence of a Phosphoribosylaminoimidazole Carboxylase-Deficient *Candida albicans* Strain in an Immunosuppressed Murine Model of Systemic Candidiasis

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The relative pathogenicities of three *Candida albicans* strains differing in the function of *ADE2* (the gene encoding phosphoribosylaminoimidazole carboxylase) were evaluated in a murine candidiasis model. *C. albicans* strain CA17 (*ade2/ade2*), previously constructed by site-specific recombination, was avirulent in immunosuppressed mice compared to the parent strain, CAF2-1, and a heterozygous *ADE2/ade2* strain obtained by transforming CA17 with a wild-type allele. The reduced virulence of CA17 was correlated with the inability to proliferate in either synthetic medium or serum without the exogenous addition of >10 µg of adenine/ml. The loss of virulence upon site-specific disruption of the *ade2* locus, and the restoration of wild-type virulence with the repair of just one *ade2* allele, confirmed that the *ADE2* gene and de novo purine biosynthesis were required for *Candida* pathogenicity. The potential of the phosphoribosylaminoimidazole carboxylase enzyme as a novel target for antifungal drug discovery is discussed.

Candida albicans is an opportunistic human pathogen causing mucosal and cutaneous infections as well as life-threatening systemic infections in immunosuppressed patients. The incidence of candidiasis has increased markedly over the past 20 years, coinciding with an increase in immunosuppressed individuals (6). This increase is primarily due to the expanded use of chemotherapy and organ transplantation and to the rapid increase in the numbers of AIDS patients. Candidiasis is currently treated with amphotericin B or with a number of drugs collectively referred to as azoles. Amphotericin B exerts its antifungal activity by binding to the fungal steroid ergosterol and disrupting membrane integrity (1), whereas the azoles directly inhibit the biosynthesis of ergosterol (9). The potential for widespread resistance to azole drugs is an increasing concern (16). The development of safe, efficacious antifungal drugs that exhibit a novel mechanism of action is an important challenge for medical research. A greater understanding of virulence mechanisms and genes required for *C. albicans* pathogenesis is needed to meet this challenge.

Recent attention has focused on the identification of virulence factors that dictate the ability of *C. albicans* to invade tissues and circumvent host defense responses. Tissue adherence (17), cell wall mannan, serine proteases, cellular hydrolases (2), and phospholipases (8) are examples of virulence factors utilized by *C. albicans* for infection. Also, a number of *C. albicans* housekeeping and morphogenesis genes are essential for *C. albicans* pathogenesis. Although not classical viru-

lence factors, these genes are critical for *C. albicans* survival in a host. For example, purine, pyrimidine, and heme biosynthesis, which are all necessary for cell growth and division in vitro, are required for *C. albicans* pathogenesis in experimental murine candidiasis (10). Similarly, myristoyl-coenzyme A:protein *N*-myristoyl transferase, which is involved in protein myristoylation (22), and the *PHR1* gene, which functions as a pH-sensitive morphological determinant (7), are essential for pathogenesis. Additional studies of both virulence genes and genes essential for *C. albicans* survival will provide a basis for future antifungal drug development.

The de novo purine biosynthetic pathway is central to cellular metabolism, providing adenine and guanine nucleotide precursors to DNA synthesis and repair, to RNA transcription, and to histidine biosynthesis. The *ADE2* gene encodes phosphoribosylaminoimidazole carboxylase, which catalyzes the sixth step of the de novo purine biosynthesis pathway. The *ADE2* gene is essential to fungi and, interestingly, *ADE2* mutants appear as red colonies when grown on agar medium (15). The red pigment is thought to result from the accumulation and subsequent polymerization of the substrate, phosphoribosylaminoimidazole (21). The de novo purine biosynthetic pathway has been associated with pathogenesis in *C. albicans*. Purine auxotrophic mutants obtained after random chemical and UV mutagenesis exhibit reduced virulence in a murine model of systemic candidiasis (12, 14, 19). These results indicate that purine auxotrophy may limit *C. albicans* virulence, with the caveat that random mutagenesis may have introduced additional unidentified mutations. Recently, the *ADE2* gene was more directly linked to reduced virulence in *C. albicans* (10) and *Cryptococcus neoformans* (13). In these studies, the *ade2* mutant strains of *C. albicans* and *C. neoformans* were less vir-

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TABLE 1. Genotypes of *C. albicans* strains

Strain	Genotype	Reference or source
CAF2-1	$\Delta ura3imm434/URA3$	4
CAI4	$\Delta ura3::imm434/\Delta ura3::imm434$	4
CAI7	$ade2::hisG-URA3-hisG/ade2::hisG \Delta ura3::imm434/\Delta ura3::imm434$	4
CAI7-R	$ADE2/ade2::hisG-URA3-hisG \Delta ura3::imm434/\Delta ura3::imm434$	This work

ulent than wild-type strains in an immunosuppressed murine model of systemic candidiasis and in an immunosuppressed rabbit model of cryptococcal meningitis, respectively. Although the *ade2* mutant strains were again generated by random mutagenesis, the reduced pathogenesis resulted directly from the *ade2* mutation since the mutant strains recovered wild-type virulence after transformation with a plasmid containing an active *ADE2* gene. These experiments more clearly defined the *ADE2* gene as essential for fungal pathogenesis.

In this report, we further investigated the role of de novo purine biosynthesis and specifically the *ADE2* gene in *C. albicans* pathogenesis. The virulence of *C. albicans* strain CAI7 (*ade2/ade2*) that was previously constructed by gene disruption (4) was evaluated in an immunosuppressed model of systemic candidiasis. The use of an *ade2/ade2* mutant strain generated by targeted gene disruption avoided the potential for secondary mutations inherent with random mutagenesis techniques. CAI7 was unable to proliferate in the kidney and was non-pathogenic compared to its parent strain, CAF2-1, and a heterozygous complemented strain, CAI7-R. The reduced virulence of CAI7 resulted directly from the block in de novo purine biosynthesis and the inability to scavenge purines from the host. Furthermore, only a single copy of the *ADE2* gene was required to restore wild-type virulence.

MATERIALS AND METHODS

Organisms and media. The yeast strains utilized for this study are listed in Table 1. The strains were routinely grown at 30°C on yeast extract-peptone-dextrose (YEPD) medium or synthetic complete medium solidified with 2% agar as needed (20). A heterozygous *ADE2/ade2* strain was constructed by repairing the *ade2::hisG* allele of strain CAI7 by homologous recombination. A *XhoI*-*NotI* restriction enzyme fragment from PBSIIzap 25 CA2 containing the *C. albicans ADE2* gene (18) was transfected into strain CAI7 spheroplasts essentially as described previously (11). The heterozygous strain, designated CAI7-R, was selected on synthetic complete medium without adenine and uridine. The *Ura*⁺ *Ade*⁺ phenotype indicated that the transfected *ADE2* DNA had recombined with the *ade2::hisG* allele and not with the *ade2::hisG-URA3-hisG* allele. The heterozygous genotype was confirmed by PCR (25 cycles; annealing temperature, 50°C) utilizing an antisense primer to the *ADE2* coding region, GGTCGATAC GAATTCTTATTTTCAATTATCAG, and a primer to the 5' promoter region, GGTCGGATCCATGGATAGCAAACTGTGG, which produced a 1.7-kb DNA fragment.

Growth rates. Strains were grown overnight in synthetic complete medium supplemented with 100 µg of adenine (Sigma)/ml. Each strain was diluted to 10⁴ cells/ml into synthetic complete medium lacking adenine. Duplicate dilutions were also made into synthetic complete medium supplemented with 100 µg of adenine/ml. Growth rates were monitored by determining both the optical density at 600 nm and the number of CFU.

Serum growth. *C. albicans* strains were grown in YEPD medium, inoculated into fresh mouse or human serum at a concentration of 10³ CFU/ml, and incubated at 35°C for up to 24 h.

Experimental murine model. Female Swiss Webster mice weighing 20 to 25 g were injected with 100 mg of cyclophosphamide (catalog no. C7397; Sigma)/kg of body weight on days 4 and 1 prior to *Candida* infection and every 3 days during the course of the experiment. Immunosuppression with this dosing regimen

FIG. 1. In vitro growth rates of CAF2-1, CAI7, and CAI7-R. Wild-type CAF2-1 (squares), *ade2/ade2* adenine auxotrophic CAI7 (circles), and heterozygous *ADE2/ade2* CAI7-R (triangles) were grown in synthetic minimal medium in the presence (closed symbols) or the absence (open symbols) of 50 µg of adenine/ml. At the designated times, growth (CFU) was determined from replicated samples diluted in YEPD broth and grown on YEPD agar for 2 days at 37°C.

was confirmed by determining the differential leukocyte counts with a Coulter counter. *Candida* strains were grown to saturation (16 h) in YEPD at 30°C. Prior to injection, yeast cells were washed three times in sterile phosphate-buffered saline, diluted, and counted on a hemacytometer. The cell concentrations were adjusted to 10⁶ to 10⁹ cells/ml for CAI7 and 10⁴ to 10⁷ cells/ml for CAF2-1 and CAI7-R. Murine candidiasis was induced by injection of 0.1 ml of yeast cell suspension into the lateral tail vein. Survival was monitored for 21 days.

FIG. 2. Virulence of *C. albicans* CAF2-1 and CAI7 in immunosuppressed mice. Mice were injected with 2×10^3 (■), 2×10^4 (●), 2×10^5 (▲), or 2×10^6 (◆) of wild-type CAF2-1 blastoconidia (top) or 5×10^5 (□), 5×10^6 (○), 5×10^7 (△), or 5×10^8 (◇) of *ade2/ade2* CAI7 blastoconidia (bottom). Ten mice were inoculated through the tail veins with the indicated number of blastoconidia, and mortality was determined over the course of a 21-day experiment. Data were recorded as percent survival on each day.

FIG. 3. Infection of *C. albicans* in the kidneys of immunosuppressed mice. Kidneys were dissected, and transverse sections were stained with H&E and GMS to aid in histological examination. Representative kidney (A) and histological (B) sections from mice 4 days after injection with 10^4 CAF2-1 (wild-type) blastoconidia are shown, as are representative kidney (C) and histological (D) sections from mice 6 days after injection with 10^5 CAI7 (*ade2/ade2*) blastoconidia.

Histological techniques. Histological analysis of the liver, lungs, spleen, and kidneys were done for lethally moribund mice or for separate groups of mice infected specifically to provide histological samples for studying time-course infection. Tissue was fixed in 10% phosphate-buffered formalin and processed with standard histological techniques. The sections were stained with hematoxylin and eosin stain (H&E), periodic acid-Schiff stain (PAS), or Gimori's methenamine silver stain (GMS) to visualize the extent of yeast cell invasion and proliferation.

RESULTS

Properties of *C. albicans* strains. Three strains of *C. albicans* were used in this study (Table 1). The parent strain, CAF2-1, was phenotypically wild type for in vitro growth and pathogenesis (5). Strain CAI7 was phenotypically an adenine auxotroph and produced red colonies on medium depleted of adenine. Strain CAI7-R was constructed by replacing the *ade2::hisG* allele of strain CAI7 with a wild-type copy of the *C. albicans ADE2* gene. Strain CAI7 was unable to grow in adenine-free medium as expected for a homozygous *ade2* mutant (Fig. 1). Interestingly, the heterozygous strain, CAI7-R, exhibited slower growth than CAF2-1 in adenine-free medium, which was reversed with exogenous adenine. The slower growth of the heterozygote indicated that purine biosynthesis was partially rate limiting for growth when only one copy of the *ade2* gene was present. All three strains had similar growth rates when

cultured in YEPD medium (data not shown) or on synthetic complete medium supplemented with 50 μ g of adenine/ml.

Systemic candidiasis in immunosuppressed mice. Invasive fungal infections typically occur in immunosuppressed hosts. We compared the virulence of strain CAI7 with that of CAF2-1, its parent strain, in an immunosuppressed murine model of candidiasis. Chronic immune suppression was achieved by administration of cyclophosphamide (100 mg/kg) intraperitoneally every 3 days, starting 4 days prior to *Candida* injection and continuing for the duration of the experiment. Lymphocyte levels were suppressed to $<100/\mu$ l for the entire course of the experiment compared to $>8,000/\mu$ l in untreated mice. The observed 50% lethal dose (LD_{50}) was 5×10^3 wild-type *C. albicans* blastoconidia per mouse for injections into the tail veins of immunosuppressed mice (Fig. 2). This was the lowest LD_{50} reported for a murine candidiasis model and likely resulted from the extended immunosuppressive treatment. Kidneys from lethally moribund or dead mice displayed numerous severe microabscesses on the surface (Fig. 3A) and exhibited prolific *Candida* invasion of the cortex, medulla, and papilla regions (Fig. 3B). In this chronic immunosuppressed murine model, microabscess formation on the kidneys enabled reliable and consistent visual scoring for *Candida* infection.

Strain CAI7 generally appeared to be nonpathogenic at

FIG. 4. Localization of *C. albicans* CAI7 in the murine lung. Immunosuppressed mice were injected with 10^8 *C. albicans* CAI7 blastoconidia through the tail veins. Lung tissues were dissected 4 days after injection. Sections were stained with PAS and GMS to aid in histological examination. Representative sections are shown for uninfected (A) and CAI7-infected (B) mice.

doses less than 10^6 blastoconidia per mouse in this model. The LD_{50} was determined to be 2.5×10^6 compared to the LD_{50} of 5×10^3 for strain CAF2- (Fig. 2). Histological examination of the kidneys provided an equally striking difference between strains. Kidney sections were examined for every mouse infected with strain CAI7 at the end of the 21-day experiment. No evidence of infection was histologically detectable in the kidney cortex or the medulla, which is the major site of infection by wild-type *C. albicans* (Fig. 3C and D). Furthermore, no secondary sites of infection were observed upon examination of heart, lung, liver, spleen, and brain tissues. Clearly, the *ADE2* gene was required for normal infection in this chronic immunosuppressed murine model.

Infection with the highest titer of strain CAI7 (5×10^8 blastoconidia/mouse) resulted in a 100% incidence of mortality in the cyclophosphamide-treated mice (Fig. 2). This mortality at the high doses appeared to be an acute response rather than the result of a progressive fungal infection, and the potential causes were investigated histologically. Mice were injected with 10^8 yeast cells of strain CAI7/mouse, and tissues were taken 1, 3, and 7 days after injection. Kidney, spleen, liver, and lung sections were stained with PAS and GMS to aid in histological

examination. At 10^8 blastoconidia/mouse, CAI7 was observed in all tissues evaluated. Yeast cells were observed in especially high numbers in the lung at 24 h after injection (Fig. 4). The microcapillary beds in the lung were highly occluded and likely resulted in the acute mortality seen at the high titer. The acute mortality and accumulation of blastoconidia in lung microcapillary beds associated with high-titer infections were also observed for strain CAI4, a nonpathogenic homozygous *C. albicans ura3* mutant, and was not specific to strain CAI7 (data not shown).

The necessity of a functional *ade2* allele for infection was further established by repairing one *ade2* allele in strain CAI7 by using site-specific homologous recombination and evaluating the virulence of the heterozygous complemented strain, CAI7-R. Strains CAF2-1 and CAI7-R were injected at 10^4 blastoconidia/mouse, and both resulted in similar rates of mortality over the course of the 21-day experiment (Fig. 5). Histological examination of kidneys from infected mice revealed that at the time of death both strains had similarly infected the kidneys, resulting in numerous microabscesses and prolific invasion of the cortex and medulla. Consistent with previous results, strain CAI7 was nonpathogenic in this experiment,

FIG. 5. Restoration of virulence for strain CAI7 in immunosuppressed mice. Mice (10 per treatment) received a tail vein injection of 10^4 blastoconidia of *C. albicans* wild-type CAF2-1 (■), heterozygous *ADE2/ade2* CAI7-R (▲), or 10^5 blastoconidia of *ade2/ade2* auxotroph CAI7 (●). Mortality was determined over the course of a 21-day experiment. Data were recorded as percent survival on each day.

even at 10^5 yeast cells/mouse. These observations demonstrated that the reduced pathogenesis of strain CAI7 resulted directly from the *ade2* mutation since full virulence was recovered by specifically repairing the *ade2::hisG* mutant allele. Furthermore, only a single functioning *ADE2* allele was required for apparent wild-type virulence.

Strain CAI7 was unable to proliferate in serum. The virulence of the mutant was attenuated presumably because serum purine levels were inadequate to support growth. To test this hypothesis, each strain was evaluated for growth in human serum. Strains CAF2-1 and CAI7-R rapidly underwent a dimorphic switch and proliferated in normal human serum in

both the presence and the absence of exogenous adenine (Fig. 6). Strain CAI7 initiated a dimorphic switch but was unable to proliferate in normal serum. The ability of strain CAI7 to proliferate in serum was restored when serum was supplemented with $50 \mu\text{g}$ of exogenous adenine/ml. Interestingly, the addition of $<10 \mu\text{g}$ of adenine/ml had little effect on CAI7 proliferation in serum, suggesting that a threshold concentration of adenine was required for growth. These experiments were repeated using murine serum with similar results (data not shown).

DISCUSSION

The de novo purine biosynthetic pathway is an essential pathway in fungi. The *ADE2* gene encodes phosphoribosylaminoimidazole carboxylase, a bifunctional enzyme that catalyzes the sixth step of de novo purine biosynthesis. Homozygous *C. albicans ade2* mutants are adenine auxotrophs in vitro and appear as red colonies due to the accumulation and spontaneous polymerization of the substrate aminoimidazole ribosyl phosphate. The requirement of the *ADE2* gene for infection was previously shown for both *C. albicans* and *C. neoformans* (10, 13) with the use of *ade2* mutants obtained by random mutagenesis procedures. Recently, a homozygous *ade2 C. albicans* strain, CAI7, was generated by targeted gene disruption, which is a direct and selective means of creating isogenic strains to study gene function. The availability of strain CAI7 provided a new opportunity to study the role of the *ADE2* gene and de novo purine biosynthesis in pathogenesis. The virulence of strain CAI7 was compared to the phenotypically wild-type parent strain, CAF2-1, from which it was derived. This was a critical experiment to determine whether strain CAI7 could scavenge purines from the host, circumventing the block in de novo biosynthesis. The titer of *C. albicans* needed to kill 50% of the mice was >100 -fold higher for strain CAI7 than for

FIG. 6. Growth of *C. albicans* in whole human serum. Strains CAF2-1 (A) and CAI7-R (B) were inoculated in whole human serum without the addition of exogenous adenine. Similar results were obtained for both strains in the presence of up to $50 \mu\text{g}$ of adenine/ml (data not shown). Strain CAI7 was inoculated into human serum with 0 (C), 0.5 (D), 5 (E), and 50 (F) μg of exogenous adenine/ml. Observations were made at 24 h after inoculation.

strain CAF2-1 in an immunosuppressed murine candidiasis model. Correlated with the reduced virulence, strain CAI7 was unable to proliferate in the cortex or medulla of the kidney, which is the primary site of infection for wild-type *C. albicans* in the murine experimental model. Furthermore, strain CAI7 was unable to infect the heart, spleen, lungs, or nervous system under the same conditions, which result in lethal systemic and disseminated candidiasis by a wild-type strain. The inability of CAI7 to proliferate in human serum was directly correlated to the observed reduced virulence. These results provided a more-detailed confirmation of a previous report that *C. albicans ade2* mutants are avirulent (10). Clearly, de novo purine biosynthesis is required for *C. albicans* infection and *ade2* mutant strains are unable to scavenge sufficient purines from host tissues to overcome the block in de novo biosynthesis.

Interestingly, strain CAI7 was not completely avirulent but instead exhibited 100-fold attenuation of virulence. Injection of 10^8 CAI7 yeast cells in the immunosuppressed murine model resulted in significant acute mortality within 48 h that was distinct from mortality resulting from the time-dependent appearance of systemic candidiasis. Acute mortality at higher doses may have reflected a limitation of the experimental animal model resulting from several possible explanations. High doses of avirulent bacterial strains often lead to acute mortality in experimental models as the result of endotoxin-induced septic shock. Although not known to produce endotoxins, *C. albicans* does produce a number of tissue-damaging proteases and hydrolases which may have contributed to the observed acute mortality at high titer (2). Additionally, the number of blastoconidia, which can be introduced into the bloodstream of mice, may have an upper limit before the shear volume of blastoconidia results in occluded capillaries. In fact, our observations confirmed that $>10^7$ CAI7 yeast cells per mouse resulted in highly occluded capillary beds in the lungs. The acute mortality resulting from tissue-degrading enzymes and physical occlusion of capillary beds likely has less clinical relevance than the time-dependent systemic infection of organs, such as the kidneys, which is observed at lower titers. When the objective is an experimental assessment of virulence or drug efficacy, we suggest that the *C. albicans* titer be more appropriately limited to ensure that the host survives the first 48 h.

C. albicans ade2 mutants exhibited some ability to survive in vivo, albeit with attenuated pathogenicity. First, *C. albicans ade2* mutants were able to undergo a dimorphic switch and initiated hyphae in serum in vitro and in murine kidney sections in vivo. Prior to the experiments, strain CAI7 was grown in rich medium containing adenine and then introduced as blastoconidia. The blastoconidia appeared to contain enough purine reserves to make a dimorphic switch and initiate hyphae. Second, some red CAI7 colonies were obtained from kidneys of chronically immunosuppressed mice up to 21 days after injection, although a majority of the kidneys were sterile at this time. The recovery of viable CAI7 colonies from minced kidneys without histological evidence of the ability to proliferate in the kidney cortex suggests that strain CAI7 may survive in a static state for several weeks.

A prediction from the murine models was that de novo purine biosynthesis and specifically the *ADE2* gene will be required for *C. albicans* infection in humans. This prediction assumes that free purine levels in human tissues are similar to

those in murine tissues; otherwise, free adenine or adenosine might circumvent a block in de novo synthesis. In fact, the *C. albicans ade2/ade2* strain was unable to proliferate in human serum unless it was supplemented with exogenous adenine. These results confirm that human serum does not have enough free purines to support growth and, in combination with the murine candidiasis experimental data, support the prediction that the *ADE2* gene is required for pathogenesis in humans. The identification of the *ADE2* gene as potentially critical for human pathogenesis has increased importance in light of recent publications that suggest a divergent catalytic mechanism between human and bacterial phosphorylribosylaminoimidazole carboxylase enzymes (3). The bacterial enzymes catalyze the ATP- and bicarbonate-dependent carboxylation of phosphorylribosylaminoimidazole to carboxyphosphoribosylaminoimidazole through an N^5 -carbamate intermediate, whereas the vertebrate *ADE2* enzymes catalyze CO_2 -dependent, ATP-independent direct carboxylation of the imidazole ring, foregoing the carbamate intermediate. Sequence analysis and complementation testing confirm that the *C. albicans ADE2* gene is similar to the bacterial genes (18). The divergent catalytic mechanisms may provide a basis to develop selective mechanism-based antifungal compounds.

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