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Candida albicans ENO1 Null Mutants Exhibit Altered Drug Susceptibility, Hyphal Formation, and Virulence

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We previously showed that the expression of *ENO1* (enolase) in the fungal pathogen *Candida albicans* is critical for cell growth. In this study, we investigate the contribution of the *ENO1* gene to virulence. We conducted our functional study of *ENO1* in *C. albicans* by constructing an *eno1/eno1* null mutant strain in which both *ENO1* alleles in the genome were knockouted with the *SAT1* flipper cassette that contains the nourseothricin-resistance marker. Although the null mutant failed to grow on synthetic media containing glucose, it was capable of growth on media containing yeast extract, peptone, and non-fermentable carbon sources. The null mutant was more susceptible to certain antifungal drugs. It also exhibited defective hyphal formation, and was avirulent in BALB/c mice.

Keywords: enolase, opportunistic human pathogen, drug susceptibility, hyphal formation, virulence

Introduction

Enolase catalyzes the conversion of 2-phospho-D-glycerate to phosphoenolpyruvate, and is critical for both glycolysis and gluconeogenesis. The enzymatic functions of enolase are highly conserved across virtually all taxa, including bacteria, plants, animals, and humans (Van der Straeten *et al.*, 1991). Enolase has alternative cellular functions. Human enolase acts as a plasminogen receptor in inflammation (Plow *et al.*, 1986; Plow and Das, 2009), and is closely associated with several disease processes, including various cancers (Nesland *et al.*, 1988; Sung and Cho, 2008). It is also a component of the avian eye lens (Rudner *et al.*, 1990; Kim and Wistow, 1993), and is a transcription repressor of the proto-oncogene c-*myc* (Feo *et al.*, 2000).

Although several *ENO1* homologues have been identified in humans, only the non-tandem enolase structural genes *ENO1* and *ENO2* are contained in the *Saccharomyces cerevisiae* genome. Mutations in one, but not both, of the *S. cerevisiae* enolase genes are viable in the presence of glucose (McAlister and Holland, 1982; Niedenthal *et al.*, 1999), indicating redundancy in enolase enzymatic activity. One explanation for the observation that mutations in both *ENO1* and *ENO2* inhibit *S. cerevisiae* from growing in the presence of glucose is glucose repression (Trumbly, 1992). DNA sequencing and southern blotting analyses in *C. albicans* indicated that only *ENO1* encodes the enolase activity (Sundstrom and Aliaga, 1992), and RNA knock-down of *ENO1* expression reduced the growth rate of *C. albicans* (De Backer *et al.*, 2001).

Enolase comprises approximately 0.7% of the total protein in yeast-form cells and 2% of that in the hyphae (Sundstrom and Aliaga, 1994). Enolase is the major antigen in candidiasis patients (Klingspor *et al.*, 1997), and it binds plasmin to induce fibrinolysis. The plasmin-bound fungal cells demonstrate increased cellular invasion (Jong *et al.*, 2003). Immunization with enolase and interleukin-12 (Montagnoli *et al.*, 2004) or β-mannan trisaccharide and enolase (Xin *et al.*, 2008) significantly reduced the kidney fungal burden in a murine model, but increased survival only moderately. In the presence of fluconazole, the expression of enolase in fluconazole-susceptible strains of *C. albicans* is substantially reduced (Angiolella *et al.*, 2002), but the amount of enolase secreted into the medium is increased (Angiolella *et al.*, 2002) compared with that in the absence of fluconazole, suggesting that enolase may be involved in mechanisms of drug susceptibility.

The mechanisms of drug susceptibility may also affect hyphal formation and virulence (Lo *et al.*, 2005). Therefore, we introduced mutations in *ENO1* in *C. albicans* to investigate the role of enolase in virulence-related processes. We used a tetracycline-regulated (TR) expression system (Baron *et al.*, 1997, 1999; Tremblay *et al.*, 1998) to investigate the function of *eno1* mutants in the *C. albicans* BWP17 strain. We constructed the *TR-ENO1/eno1* strain, in which one copy of the *ENO1* gene is replaced by the *ARG4* selective nutrition marker and the other is under the control of a TR promoter (Nakayama *et al.*, 2000). We showed that the expression of *ENO1* in *C. albicans* is critical to cell growth in glucose-containing media (Yang *et al.*, 2006a).

Our TR expression system is, however, suboptimal for the evaluation of the null phenotype because mutant phenotypes may be affected by the use of nutrient markers in gene-replacement experiments (Chibana *et al.*, 2005; Sharkey *et al.*, 2005). And, in the presence of doxycycline, the metabolic activity of the *C. albicans* biofilm was reduced by as much as 85%, and the combined high levels of doxycycline and fluconazole demonstrated synergistic antifungal activity

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(Miceli *et al.*, 2009). Thus, we have constructed an *eno1/eno1* null mutant in the SC5314 strain of *C. albicans* using the drug-resistant *SAT1* flipper cassette, which contains the nourseothricin-resistance marker (Reuss *et al.*, 2004). We found that, although the growth of the null mutant was unaffected on media containing glycerol, yeast extract, and peptone, the null mutations rendered the cells incapable of growing in the presence of glucose. We also observed that the null mutations of *ENO1* in *C. albicans* affected drug resistance, hyphal formation, and virulence.

Materials and Methods

Media

Media materials were purchased from Difco Laboratories (BD Biosciences, USA). Various selective synthetic media (SM) were prepared, which contained a 0.67% yeast nitrogen base (without amino acids) supplemented with 2% dextrose (SD), 2% ethanol (SE), 2% galactose (SGal), 2% glycerol (SG), 3% glycerol and 2% ethanol (SGE), 100 mM pyruvate (SP), or 2% dextrose and 100 mM pyruvate (SDP). Additional selective yeast-peptone media (YP) were prepared, which contained 1% yeast extract and 2% peptone supplemented with 2% dextrose (YPD), 2% ethanol (YPE), 2% galactose (YPGal), 2% glycerol (YPG), 3% glycerol and 2% ethanol (YPGE), 100 mM pyruvate (YPP), or 2% dextrose, and 100 mM pyruvate (YPDP), as previously described (Sherman, 2002). Agar media were prepared by adding 2% agar to the various selective media.

Construction of the *eno1*/*eno1* null mutant strain

The function of the *ENO1* gene in the SC5314 strain of *C. albicans* was disrupted by the insertion of the *SAT1* flipper cassette into *ENO1* using a previously described method (Reuss *et al.*, 2004). The *ENO1* upstream DNA sequences from nucleotide positions -578 to -50 were amplified during polymerase chain reaction (PCR) using the HJL980 primer (5′-ggtaccATTAAGCCGTGGGTTCTCAA-3′) and the HJL981 primer (5′-ctcgagAAAAAGGGAGAAAAGGAAAGAAA-3′). The *ENO1* downstream DNA sequences from nucleotide positions of 1308 to 1884 were PCR amplified using the HJL982 primer (5′-GGCTTCTCAATTGTAAGTTTGC-3′) and the HJL983 primer (5′-CAGGATCTATTGACGAATT CCA-3′). The PCR products were cloned into the pGEM-T Easy plasmid (Promega, USA), and competent *Escherichia coli* cells were transformed with the ligated products. The downstream sequences were excised from purified plasmid DNA using the *Not*I restriction endonuclease, and the upstream sequences were excised using *Kpn*I and *Xho*I. The upstream and downstream sequences of *ENO1* were cloned at the termini of the *SAT1* flipper cassette in the pSFS2A plasmid to produce the LOB317 plasmid. The DNA of LOB317 which was subjected to *Kpn*I and *Sac*I to produce the DNA fragment containing the *SAT1* flipper cassette flanked by the upstream and downstream *ENO*1 sequences for the transformation of SC5314.

The wild-type SC5314 strain of *C. albicans* was transformed by replacing the corresponding genomic *ENO1* sequences with the LOB317 *SAT1* flipper through a double cross-over in a 2-step procedure. After transformed with the LOB317 fragment, the transformants were plated onto YPD containing 200 μg/ml of nourseothricin. The nourseothricin-resistant colonies were grown on YP with 2% maltose for 48 h to induce the FLP recombinase-mediated excision of the *SAT1* flipper cassette to obtain the nourseothricin sensitive, heterozygous *ENO1/eno1* mutant strain YLO365. The homozygous *eno1/eno1* null mutant strain YLO367 was constructed by repeating the procedure following the excision of the second wild-type *ENO1* allele from the YLO365 strain, except that the selection was performed on YP agar with glycerol and nourseothricin.

The *ENO1* gene was reintroduced into the *eno1/eno1* null mutant genome to obtain the rescued strain by PCR amplification of the gene using the HJL980 primer (5′-ggtaccAT TAAGCCGTGGGTTCTCAA) and the HJL983 primer (5′-CAGGATCTATTGACGAATTCCA), nucleotide positions -578 to 1884, and the PCR product was cloned into the pGEM-T plasmid. The wild-type *ENO1* gene sequences were excised from pGEM-T as a single fragment using *Kpn*I and *Xho*I, and was cloned into LOB317 through the *Kpn*I and *Xho*I sites, replacing the upstream fragment to generate the LOB318 plasmid. The DNA of LOB318 was cleaved with *Kpn*I and *Sac*I, and the resulting fragment that contained the *ENO1* and the *SAT1* flipper cassette was transformed into YLO367. The transformants were plated on YPD with nourseothricin. The nourseothricin-resistant colonies were cultured on YP with 2% maltose for 48 h to induce the FLP-mediated excision of the *SAT1* flipper cassette to generate the *eno1/eno1::ENO1* rescued strain YLO369.

Southern blot analysis

A single YLO369 colony was selected for southern blot analysis to confirm the presence of the recombinant *ENO1* gene in the YLO369 genome. The genomic DNA was isolated and digested with *Acc*I. The digested genomic DNA fragments were separated by electrophoresis on a 1.2% agarose gel, and transferred to a nylon membrane. The *ENO1* DNA sequence between nucleotide positions 1308 and 1884 relative to the translation initiation site was used as the template to synthesize the DIG-labeled probe (Roche Diagnostics, USA) for the southern blotting analysis, according to the manufacturer instructions.

Antifungal susceptibility tests

A broth microdilution method (Clinical Laboratory Standards Institute, 1997) was used to examine the susceptibility tests using YPGE media and the following concentration ranges of antifungal agents: amphotericin B (0.0625 to 4 μg/ml), calcofluor white (10 to 640 μg/ml), fluconazole (0.125 to 8 μg/ml), miconazole (0.156 to 10 μg/ml), and voriconazole (0.004 to 0.25 μg/ml). Cell growth was determined based on the optical density of the media that was measured using a Biotrak II plate reader (Amersham Biosciences, USA) after incubation at 35°C for 48 h.

Mouse model for virulence

The mouse model experiments used to test the virulence of

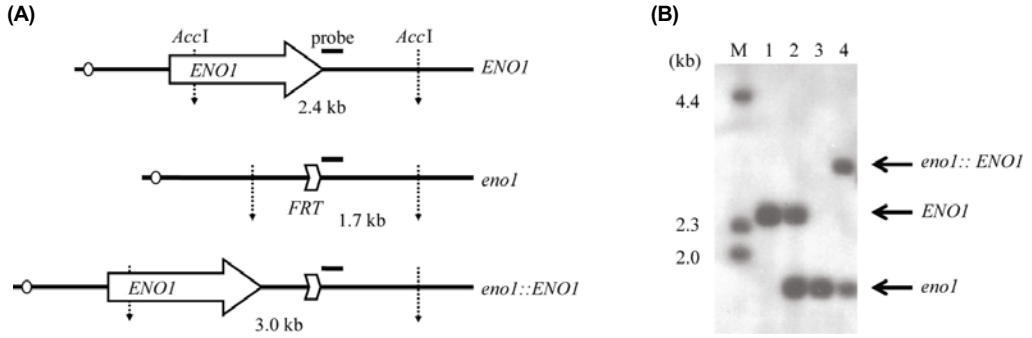

Fig. 1. Construction of the *ENO1* mutant strains of *C. albicans*. (A) Diagram for the construction of the mutant strains. Both copies of the *ENO1* were replaced by *SAT1* flipper cassettes to produce the *eno1/eno1* null mutant. A wild-type allele of *ENO1* was reintroduced into the null mutant to produce the *eno1/ eno1::ENO1* rescued strain. Arrows represent the *Acc*I restriction enzyme sites. The thick horizontal bar indicates the location of the probe for the southern blot analysis. (B) Genomic DNA digested with *Acc*I was analyzed by southern blotting. Lanes: 1, SC5314; 2, *ENO1/eno1* (YLO365); 3, *eno1/eno1* (YLO367); 4, *eno1/eno1::ENO1* (YLO369). The molecular weight standards (Lane M) are indicated (left).

the different *C. albicans* strains were conducted as previously described (Lo *et al*., 1997; Chen *et al*., 2006). Male BALB/c mice (18 to 20 g) were obtained from the National Laboratory Animal Center (Taipei, Taiwan). The animal studies were performed in accordance with the NIH Guide to the Care and Use of Laboratory Animals and the Animal Welfare Act in an AAALAC-accredited facility. All animal protocols were approved by the Institutional Animal Care and Use Committee at the National Health Research Institutes.

Each strain was tested for virulence by the injection of 0.5 ml of the cell suspension (approximately 1×10^6 cells/mouse) into the tail vein. The virulence assessment was performed using the wild-type SC5314 cells, the *eno1/eno1::ENO1* rescued cells, and the *eno1/eno1* null mutant cells in 10, 10, and 15 mice, respectively. To determine the kidney fungal burden at different stages of infection, we recovered the left kidney from 2, 3, and 10 mice on the third, fourth, and 20th day, respectively, following inoculation. The kidney from each mouse was homogenized, and the number of *C. albicans* cells in the homogenate was determined by scoring the cells in a hemocytometer and culturing the cell solutions on agar plates to determine the viable count.

Growth curve

Approximately 1×10^4 *C. albicans* cells were cultured on the appropriate media in bio-screen plates, and the Bioscreen C analyzer (Oy Growth Curves AB, Finland) was used to assess the growth of the different isolates.

Results

Construction of the *eno1/eno1* null mutant with the *SAT1* flipper cassette

The genomic DNA from the SC5314 strain and the various mutant strains was analyzed using southern blotting to assess the mutagenesis. The blot of the SC5314 strain showed a single band that corresponded to a molecular weight slightly higher than the 2.3-kb molecular weight standard (Fig. 1), which is consistent with the 2.4-kb *ENO1* genomic fragment. The blot of the heterozygous knockout strain showed the 2.4-kb *ENO1* band and the 1.7-kb fragment of the *eno1* knockout construct. The blot of the homozygous knockout strain *eno1/enol* showed a single 1.7-kb band. The blot of the rescued strain showed a band that corresponded to a molecular weight between 2.3 and 4 kb, which was consistent with the 3-kb *eno1::ENO1* rescue allele.

eno1/eno1 null mutant cannot metabolize glucose

The *eno1/enol* null mutant failed to grow in the presence of glucose, whereas the growth of the SC5314 and rescued strains was not inhibited (Fig. 2, columns B, C, and I). The growth of the null mutant was also inhibited to a lesser extent on the YP media, compared with that of the SC5314 strain. The SC5314 strain grew on the YPGE, YPG, and YPGal selective media (Fig. 2, columns F, E, and G). The growth of the null mutant on YPP and YPE was similar to that observed in the YP medium (Fig. 2, columns H and D).

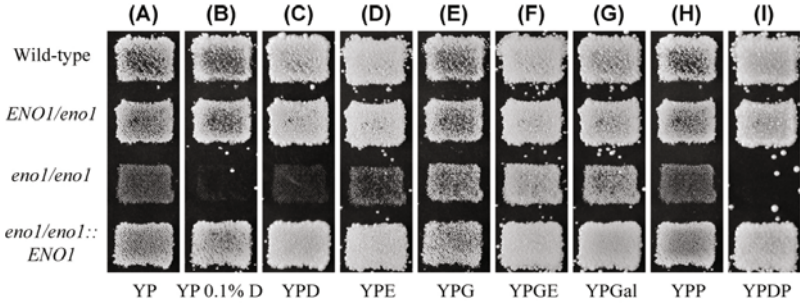

Fig. 2. Enolase is required for usage of glucose in the *eno1/eno1* null mutant of *C. albicans*. The SC5314, *ENO1/ eno1* (YLO365), *eno1/eno1* (YLO367), *eno1/eno1::ENO1* (YLO369) strains were cultured on YPG media at 30°C for 48 h, before being replica-plated onto different media. The replicates were photographed after incubation at 30°C for 48 h. The media contained the following components: yeast peptone (YP, 1% yeast extract and 2% peptone) with 2% dextrose (YPD), 2% ethanol (YPE), 2% galactose (YPGal), 2% glycerol (YPG), 2% ethanol, and 3% glycerol (YPEG), 100 mM pyruvate (YPP), or 2% dextrose and 100 mM pyruvate (YPDP).

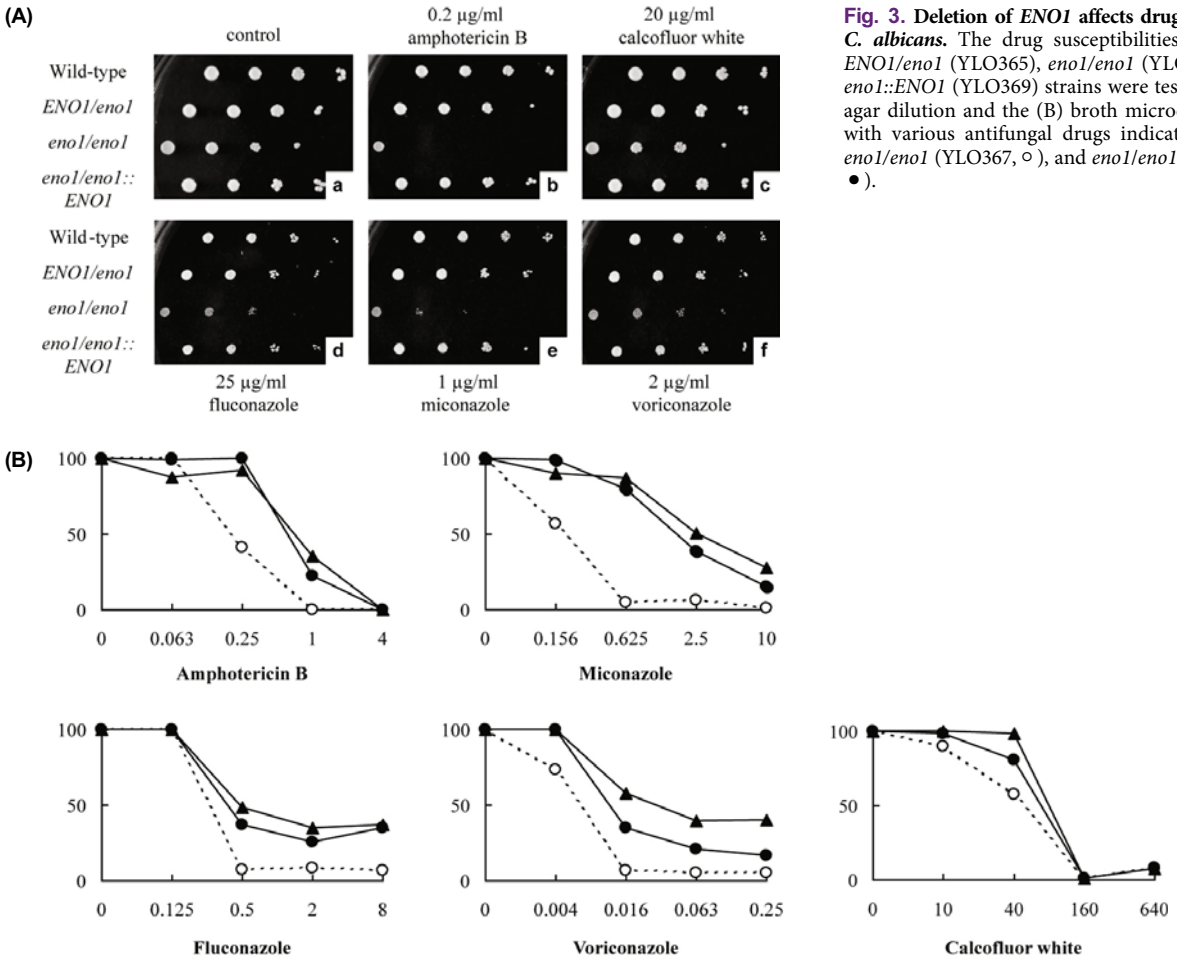

Fig. 3. Deletion of *ENO1* affects drug susceptibility in *C. albicans*. The drug susceptibilities of the SC5314, *ENO1/eno1* (YLO365), *eno1/eno1* (YLO367), and *eno1/eno1::ENO1* (YLO369) strains were tested using the (A) agar dilution and the (B) broth microdilution methods with various antifungal drugs indicated. SC5314 (▲), *eno1/eno1* (YLO367, ○), and *eno1/eno1::ENO1* (YLO369, ●).

The SC5314 strain also grew in liquid SM with various carbon sources, but the null mutant did not (data not shown). Thus, the *eno1/eno1* null mutant was able to use only the components of the YP media as a carbon source.

eno1/eno1 null mutants exhibit increase drug susceptibility

To determine whether enolase is involved in drug susceptibility, we performed drug susceptibility tests using the agar dilution method. All cells grew on YPGE media without drugs (Fig. 3Aa). The number of *eno1/eno1* null mutant cells that grew on media containing amphotericin B (Fig. 3Ab) or miconazole (Fig. 3Ae) was significantly less than that of the SC5314 and rescued strains. To further test the effects of the null mutations, drug susceptibility was also evaluated using the broth microdilution method to minimize the potential growth differences in these strains. The growth of each strain in the presence of the various drugs was normalized

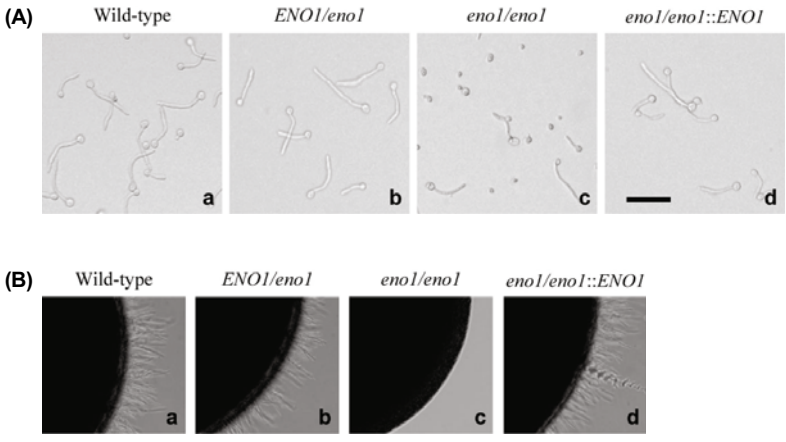

Fig. 4. Deletion of *ENO1* affects hyphal formation in *C. albicans*. (A) The SC5314, *ENO1/eno1* (YLO365), *eno1/eno1* (YLO367), and *eno1/eno1::ENO1* (YLO369) cells were cultured in YPG liquid media (1% yeast extract, 2% peptone, and 2% glycerol) to monitor germ tube formation. Photographs were taken after incubation at 37°C for 2 h. (B) The SC5314, *ENO1/eno1* (YLO365), *eno1/eno1* (YLO367), and *eno1/eno1::ENO1* (YLO369) cells were cultured on YPG agar plates to monitor the hyphal formation in colonies. Photographs were taken after incubation at 30°C for 60 h.

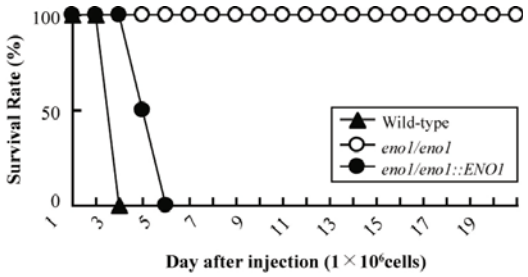

Fig. 5. Deletion of *ENO1* diminishes the virulence of *C. albicans* in a mouse model. BALB/c mice were injected with approximately 1×10^6 cells of the SC5314 (▲), *eno1/eno1* (YLO367, ○), and *eno1/eno1::ENO1* (YLO369, ●) strains separately, and mouse survival was monitored daily.

to that in the absence of drugs. The null mutant exhibited different degrees of susceptibility to the various antifungal drugs, compared with the SC5314 and the rescued strains (Fig. 3B, broken lines vs. solid lines). The deletion of *ENO1* caused increased susceptibility to amphotericin B and miconazole, and increased susceptibility to fluconazole, voriconazole, and calcofluor white were also observed to a lesser extent (Figs. 3A and 3B).

eno1/eno1 null mutants exhibit reduced hyphal growth

Consistent with the previous report that glycerol induces hyphal growth (Arguelles *et al*., 1999), the SC5314 cells formed germ tubes in the YPG medium (Fig. 4Aa). The *eno1/eno1* null mutant cells exhibited reduced germ-tube formation, compared with that in the SC5314 strain, suggesting that *ENO1* contributes to germ tube formation (Fig. 4Ac). The contribution of enolase to hyphal growth was also evident on examination of the colony morphology (Fig. 4B). Along the edge of the SC5314 colonies, hyphae were clearly visible (Fig. 4Ba), whereas none were observed for the null mutant colonies (Fig. 4Bc). The re-introduction of the *ENO1* allele restored hyphal formation (Fig. 4Bd).

eno1/eno1 null mutants exhibit diminished virulence *in vivo*

To investigate whether the reduced hyphal formation and the altered glucose metabolism observed for the null mutant contribute to diminished virulence *in vivo*, we evaluated the virulence of the null mutant in a murine model of systemic infection, and compared it with that of the SC5314 and rescued strains. The mice infected with the *eno1/eno1* null mutant cells survived longer than 20 d following inoculation (Fig. 5, open circles). In contrast, mice infected with the SC5314 strain (Fig. 5, solid triangles) or the *eno1/no1::ENO1* rescued strain (Fig. 5, solid circles) survived less than 5 d after inoculation. We also examined the kidney fungal burden in infected mice. The homogenates of kidneys from mice infected with the SC5314 or the rescued strain yielded a mean of 3.01×10^6 and 6.82×10^6 CFU/g, respectively, whereas the kidney homogenates from mice inoculated with the null mutant cells produced no colonies.

Discussion

Enolase has been proposed to contribute to various processes in *C. albicans* (Klingspor *et al*., 1997; Angiolella *et al*., 2002; Jong *et al*., 2003; Yang *et al*., 2006a). Using tetracycline-regulated *ENO1* expression in the *TR-ENO1/eno1* strain, we previously showed that the expression of *ENO1* in *C. albicans* is critical for cell growth in the presence of glucose, but not for the catabolism of non-fermentable carbon sources. However, the regulation of *ENO1* expression in the *TR-ENO1/eno1* strain requires doxycycline, which may affect fungal growth, especially in the presence of fluconazole (Miceli *et al*., 2009).

Gene replacement in the *TR-ENO1/eno1* strain was performed using nutrition-based selective markers (Lo *et al*., 2005; Yang *et al*., 2006b; Chen *et al*., 2009), and there are growing concerns regarding the effects of such markers on mutant phenotypes (Chibana *et al*., 2005; Sharkey *et al*., 2005). Thus, we used the *SAT1* flipper cassette to perform the *ENO1* gene replacement because it allows for selection based on nourseothricin resistance. We constructed the *eno1/eno1* null mutant from the SC5314 strain to investigate the functions of enolase without the potential influence of doxycycline on cell growth and the effects of nutritional markers on the null mutant phenotype. Our results show that enolase in *C. albicans* is involved in drug susceptibility, hyphal growth, and virulence.

Enolase catalyzes the conversion of 2-phospho-D-glycerate to phosphenolpyruvate in the glycolytic pathway (Jong *et al*., 2003), and thus, is essential to both glycolysis and gluconeogenesis. Despite the nullification of *ENO1* function, the null mutant was capable of growth on YP, albeit at a lower rate than that of the SC5314 strain. Thus, the YP medium was sufficient as an energy source for gluconeogenesis and glycolysis in the null mutant. However, the growth of the null mutant on YP was completely inhibited when glucose was present, an observation that is consistent with glucose suppression (Mason *et al*., 1993). We also found that the null mutant was unable to grow in SM, regardless of the carbon source supplied, which is inconsistent with previous reports that the null mutant can use glycerol as a carbon source through sequential conversion to glycerol-3-phosphate, glycerone phosphate, and fructose-1,6-phosphate by aldolase (Porter *et al*., 2001) or conversion to acetyl-coA as a substrate for the tricarboxylic acid cycle (Sorger and Daum, 2003).

Blood glucose concentrations range from 0.075% to 0.1% in humans and 0.1% to 0.12% in mice (Keren *et al*., 2000; Tsuneki *et al*., 2004). In our present study, the *eno1/eno1* null mutant failed to grow in a media containing 0.1% glucose (Fig. 2B), which suggests that the *eno1/eno1* null mutant was not virulent in the mouse model because glucose is present in blood. Our *in vivo* experiments showed that no null mutant cells were detected in the kidneys of previously inoculated mice (Fig. 5), indicating that the null mutant was unable to proliferate in the mouse body, an observation that differs from that of other avirulent strains of *C. albicans* (Chen *et al*., 2006; Yang *et al*., 2009).

The null mutant displayed various degrees of increased susceptibility to antifungal drugs, compared with the SC5314 strain (Fig. 3), which suggests that *ENO1* plays a role in drug susceptibility. In addition, despite its ability to form colonies, the null mutant was unable to form hyphae on YPG agar plates (Fig. 4B). Thus, its inability to form germ tubes

in liquid media (Fig. 4A) was not due to cell death. Enolase is one of the major antigenic determinants in the host (Eroles *et al.*, 1997; Pitarch *et al.*, 2004). It would, therefore, be interesting to explore whether the host immune system may respond to the null mutant cells.

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